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A NEW METHOD OF DETERMINING CLAY CONTENT OF SOILS BY MOISTURE ABSORPTION AT 70 PER CENT HUMIDITY

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Received for publication October 29, 1931

The conception of hygroscopic coefficient, i.e., the percentage of moisture taken up by dry soil from an atmosphere saturated with water vapors, is an old one; and at one time was regarded as a very important constant for characterizing a particular soil. The interest in it received a fresh impetus from the researches of Briggs and Shantz, who gave empirical formulas correlating hygroscopic coefficient with wilting coefficient as well as the mechanical composition of soils (1). It was soon recognized, however, that these empirical formulas had only a limited applicability, and only a rough correlation between mechanical composition and hygroscopicity was all that could be reasonably expected.

The difficulty in finding a more rigid relationship lay in the very nature of things. Methods of determining clay (which is mainly responsible for moisture absorption) as well as hygroscopic coefficient were extremely faulty. With the introduction of newer methods of mechanical analysis of soils clay can be determined with great precision; but hygroscopic coefficient still remains a factor easy to imagine theoretically, but most difficult to attain in practice, even when the technique is refined considerably beyond that reasonably possible in routine determinations (3).

The difficulty of maintaining a saturated atmosphere for any length of time led Mitscherlich to suggest (2) that hygroscopicity of the soil should be determined by bringing it into equilibrium with the partial pressure of water vapor afforded by 10 per cent H_2SO_4 (i.e. 96 per cent humidity). Somewhat similar considerations led the U. S. Bureau of Soils workers to recommend 3.3 per cent H_2SO_4 (i.e. 99 per cent humidity), for the same purpose. It must be admitted, however, that these concentrations are fixed arbitrarily, and there is no valid ground for the belief that they represent any fundamental advance on the original conception of the hygroscopic coefficient.

The first important step forward in our knowledge regarding hygroscopicity of soils is due to Thomas (9) who measured the vapor pressure of soils at differ-

[1] This work was brought to completion by the help of a grant from the Imperial Council of Agricultural Research, and the writer takes this opportunity of recording his indebtedness to the council. Acknowledgments are also due to the Department of Agriculture, Punjab, for the loan of apparatus and other facilities for work.

ent moisture contents. This was followed by the work of Puri et al. (8) on the relation between vapor pressure and moisture content of soils, in which the suggestion was put forward that hygroscopicity of soils should be measured by bringing it into equilibrium with an atmosphere of 50 per cent humidity, as this occurred on the point of inflection of vapor pressure-moisture content curve and any error in controlling vapor pressure would only result in a small difference in equilibrium moisture content.

Another difficulty about any moisture absorption experiment is the definition of "dry" soil. The usual practice is to dry in an oven at 100–105°C. This has the advantage of rapidity but little confidence can be placed in it. Drying over H_2SO_4 is to be preferred, though the process is slow. Both of these methods are frequently attended with permanent changes in the soil

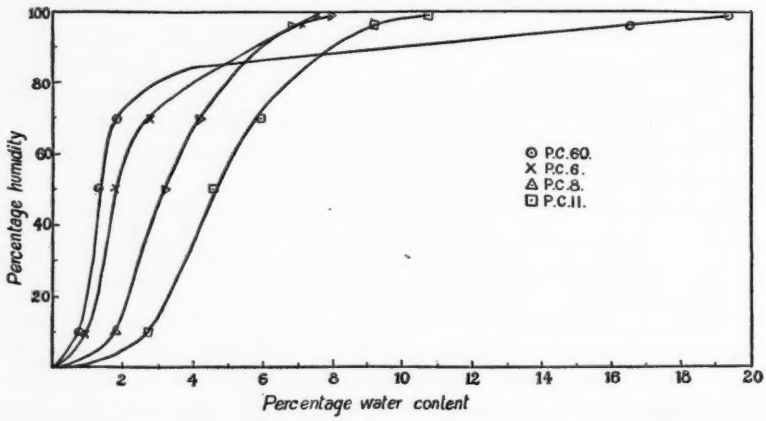

FIG. 1. TYPICAL VAPOR PRESSURE CURVES OF TWO NORMAL SOILS (P.C. 8 and 11), A HIGHLY ACID SOIL (P.C. 6), AND A HIGHLY ALKALINE SOIL (P.C. 60)

colloids. In order to get over this difficulty the author has suggested elsewhere (6) that since the interval between 10 per cent and 70 per cent humidity on a vapor pressure curve for soil is sensibly straight (fig. 1), this should be made the basis of expressing hygroscopicity of soils. It was also shown that the influence of exchangeable ions in soil colloids is felt the least in this portion of the curve and by adopting it as a "single value" we shall be expressing the specific surface of the soil particles, irrespective of the nature and extent of the extraneous influences that generally come into play in soils.

A great feature of the vapor pressure curve of soils is that normal soils generally give normal curves, whereas abnormal curves are almost invariably obtained with highly acid or highly alkaline (especially those containing exchangeable Na or free Na_2CO_3) soils (fig. 1), but for all soils the portion between 10–70 per cent humidity is the least affected.

It will be clear from the nature of the curves shown in figure 1 that the hitherto considered "dry point" is quite as indefinite as the equilibrium point with saturated atmosphere. By adopting 10 per cent humidity equilibrium value as the "dry" basis, we can be sure of a greater precision in the determination of this reference point. On this basis the vapor pressure curve up to 70 per cent humidity being a straight line passing through the origin, the determination of a single value will enable us to interpolate hygroscopicity at any other humidity between 10 and 70 per cent.

Since hygroscopicity (10–70 per cent humidity) is only slightly affected by the nature of soil colloids, it should show a better correlation with clay content in different soils. This is actually the case. Indeed it is possible to calculate the percentage of clay (0.001 mm. diameter) in a given soil by determining its hygroscopicity in this manner (table 1).

It is to be noted that the hygroscopicity values for a given humidity are higher when that humidity has been reached by drying than when it is reached. by wetting (8). Both the values were determined for the 57 soils examined. The correlation coefficient when the equilibrium values were reached by drying was found to be 0.996, and when they were reached by wetting the correlation was 0.993. Both these values are highly significant. The following relations hold good between clay (0.001 mm.) and hygroscopicity, (H).

Drying

$$\text{Clay} = 8.04\,H + 1.02$$

Wetting

$$\text{Clay} = 8.41\,H + 2.8$$

In table 1 the calculated values of clay are the mean of the drying and wetting hygroscopicity values. The agreement between the calculated values and those found by direct determination with the pipette method, using the NaCl-NaOH method of dispersion described by the author elsewhere (5), is as good as could be expected from a highly complex body like soil, when the errors involved in the measurements are considered. The few soils that show rather serious discrepancy are invariably abnormal soils. Soil P.C. 6 is a highly acid soil entirely barren, P.C. soils 53, 56, 57, 58 and 60 are highly alkaline barren soils containing free Na_2CO_3.

Absorption of moisture from humidities higher than 70 per cent is largely affected by the state of aggregation of the soil colloids. It would appear therefore that the amount of moisture absorbed by a soil between 70–99 per cent humidity will indicate characteristic differences between soil colloids from various sources depending on their state of aggregation. However, to reduce such moisture absorption to a common denominator in all soils the water thus absorbed should be divided by hygroscopicity (10–70 per cent humidity) which has been shown to be a function of the total clay content. Table 1 also includes these values for 58 soils. The various soils have been arranged according to the province from which they were obtained. It will be seen that on the whole soils from any particular province show approximately the same (70–

TABLE 1

*The relation between hygroscopicity, clay content, and dispersion coefficient**

	SOIL	H	CLAY (0.001 MM.)		DIFFERENCE	$\frac{H'}{H}$	D.C.
			Calculated	Found			
		per cent	*per cent*	*per cent*	*per cent*		*per cent*
Punjab soils	P.C. 4	0.79	8.4	10.5	+2.1	2.96	23.7
	P.C. 5	0.56	6.5	9.6	+3.1	3.23	30.8
	P.C. 7	1.95	17.9	17.5	−0.4	7.48	94.7
	P.C. 16	0.52	6.1	5.4	−0.7	4.02	20.7
	P.C. 34	0.68	7.4	8.1	+0.7	2.40	7.1
	P.C. 35	1.75	16.3	16.9	+0.6	0.90	4.9
	P.C. 36	0.74	8.0	9.0	+1.0	2.39	5.1
	P.C. 47	1.01	10.2	12.8	+2.6	3.49	57.9
	P.C. 48	1.10	10.9	15.7	+4.8	3.68	9.6
	P.C. 53	0.85	8.8	15.2	+6.4	3.36	61.3
	P.C. 54	0.15	3.1	2.6	−0.5	4.67	20.2
U. P. soils	P.C. 17	1.00	10.1	8.9	−1.2	3.84	8.5
	P.C. 24	0.83	8.7	5.5	−3.2	3.28	4.5
	P.C. 57	0.60	6.8	1.9	−4.9	3.10	49.4
	P.C. 58	2.12	19.3	11.7	−7.6	7.72	100
	P.C. 59	0.81	8.6	7.8	−0.8	1.93	26.5
	P.C. 60	1.24	12.2	4.8	−7.4	14.40	92.3
C. P. soils	P.C. 13	6.32	53.9	56.5	+2.6	1.28	6.4
	P.C. 28	4.35	37.7	42.8	+5.1	1.22	1.8
	P.C. 29	6.34	54.0	61.1	+7.1	1.31	2.9
	P.C. 30	6.72	57.1	53.5	−3.6	0.96	2.7
Bombay soils	P.C. 2	6.04	51.6	56.1	+4.5	1.33	2.7
	P.C. 3	6.69	56.7	61.2	+4.5	1.16	3.7
	P.C. 38	5.59	47.8	50.9	+3.1	1.17	2.3
	P.C. 39	1.30	12.5	7.2	−5.3	1.44	5.9
	P.C. 40	1.26	12.3	11.6	−0.7	1.52	2.3
	P.C. 41	6.39	54.4	51.6	−2.8	1.11	2.4
	P.C. 42	7.01	59.5	54.4	−5.1	1.28	2.2
	P.C. 46	6.58	56.0	55.9	−0.1	1.27	1.6
Bihar soils	P.C. 1	0.31	4.5	6.4	+1.9	5.52	26.5
	P.C. 43	1.90	17.5	16.2	−1.3	3.07	7.4
	P.C. 44	0.37	5.0	5.4	+0.4	4.68	5.9
	P.C. 45	0.78	8.3	8.9	+0.5	2.24	5.4
	P.C. 52	0.82	8.6	10.5	+0.9	2.37	6.2
Bengal soils	P.C. 6	1.79	16.6	25.2	+8.6	2.87	4.2
	P.C. 37	0.45	5.6	2.8	−2.8	2.47	22.9
	P.C. 49	2.73	24.3	26.7	+2.4	1.68	2.6

TABLE 1—*Concluded*

	SOIL	H	CLAY (0.001 MM.)		DIFFERENCE	$\frac{H'}{H}$	D.C.
			Calculated	Found			
		per cent	*per cent*	*per cent*	*per cent*		*per cent*
Madras soils	P.C. 8	2.59	23.2	22.5	−0.7	1.42	4.0
	P.C. 9	1.51	14.3	15.9	+1.6	4.13	4.6
	P.C. 10	3.79	33.0	32.2	−0.8	1.50	2.8
	P.C. 11	3.26	28.6	29.3	+0.7	1.49	3.2
	P.C. 14	2.51	22.5	16.6	−5.9	2.98	27.9
Assam soils	P.C. 12	0.60	6.8	8.8	+2.0	9.62	9.7
	P.C. 15	1.92	17.6	17.2	−0.4	4.13	5.1
Burma soils	P.C. 18	1.35	13.2	15.1	+1.9	2.98	17.1
	P.C. 20	0.34	4.7	5.3	+0.6	5.15	8.6
	P.C. 21	1.33	12.8	9.7	−3.1	2.42	8.6
	P.C. 22	1.02	10.3	11.9	+1.6	2.55	5.3
	P.C. 23	0.98	9.5	8.9	−0.6	2.18	6.4
Travancore soils	P.C. 31	2.17	19.7	20.9	+1.2	1.64	1.3
	P.C. 32	6.81	57.7	56.3	−1.4	3.07	3.4
Mysore soils	P.C. 26	3.11	27.4	22.4	−5.0	0.89	3.1
	P.C. 27	6.35	54.1	52.6	−1.5	1.17	2.3
Sind soils	P.C. 55	0.96	9.8	6.8	−3.0	3.98	15.3
	P.C. 56	1.37	13.2	8.7	−4.5	5.93	20.6
N. W. F. soils	P.C. 50	1.47	13.9	13.8	−0.1	2.55	11.3
	P.C. 51	1.08	10.7	9.7	−1.0	2.76	14.7

* H = hygroscopicity between 10–70 per cent humidity.
H′ = hygroscopicity between 70–99 per cent humidity.
D. C. = Dispersion coefficient.

99 per cent) hygroscopicity per unit surface area with here and there abnormally high values the significance of which will be understood from what follows:

Moisture from the vapor phase may be absorbed by soils in three ways. It may enter into loose chemical combination and exist as water of hydration; it may be taken up in the minute capillaries on the surface of individual particles (2); or it may fill up the interstitial spaces between the aggregates of various particles. It is conceivable that the portion of the vapor pressure curve below 10 per cent humidity (fig. 1) represents water of hydration; between 10–70 per cent humidity, capillary adsorbed water and the portion above interstitial water. Water of hydration will depend on the constitutional nature of soil colloids, the capillary absorbed water on the extent of their surface area, and interstitial water on their state of aggregation. The cor-

rectness of this hypothesis in the case of capillary absorbed water is shown by the fact that this moisture is correlated with the total clay content. In the case of interstitial water it is supported by the fact that we have an independent method of determining the state of aggregation of the soil particles, i.e., the measurement of dispersion coefficient (7), and as is seen from table 1, soils in general show a positive correlation between interstitial water per unit surface and dispersion coefficient. The correlation coefficient works out to be 0.765, which is highly significant. The method of determining dispersion coefficient may be briefly stated here:

Ten grams of soil is made up to one liter with water and left overnight in a cylinder or bottle of uniform diameter, after the soil and water have been mixed by turning the bottle end over end a few times, taking care that the suspension is not subjected to any violent agitation which might break up the crumbs. Next morning the suspension is again thoroughly mixed by gentle end over end motion and allowed to settle for the appropriate time, and clay (0.002 mm.) is determined by pipetting from the proper depth exactly as in the pipette method of mechanical analysis. The clay thus obtained is called the dispersion factor (D.F.). The total clay (C) obtainable on complete dispersion of the soil is determined by the author's NaCl-NaOH method (5). Dispersion coefficient (D.C.) is calculated by the formula

$$\text{D.C.} = \frac{\text{D.F.} \times 100}{\text{C}}$$

A sharp distinction between the three forms of hygroscopic moisture is not possible by the very nature of things. For one thing there are no sharp breaks in the vapor pressure curve; but from an examination of these curves it may be safely stated that fixing 10–70 per cent humidity limits is not altogether arbitrary, and will eventually help us in elucidating the mechanism of moisture absorption much better than the simpler conception of the hygroscopic moisture as a uniform film of water round the soil particles.

SUMMARY

The vapor pressure curve for soils could be split up into three portions representing three forms of hygroscopic moisture i.e. hydration, capillary absorbed, and interstitial. The first depends on the constitutional nature of soil colloids, the second on their surface area, the third on their state of aggregation.

Though a sharp distinction could not be made between the three forms of moisture, there is evidence to show that we shall not be far wrong in considering 10 per cent humidity as marking the end of the first form, and 70 per cent humidity as the end of second form.

Clay (0.001 mm.) content of a soil can be estimated from moisture absorption between 10–70 per cent humidity with a fair amount of accuracy, by the help of an empirical formula.

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IRON IN RELATION TO THE STIMULATION OF GROWTH BY HUMIC ACID[1]

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Humic acid stimulates the rates of growth of various higher and lower plants. Many attempts have been made to determine the cause of its beneficial action. Kaserer (25, 26) suggested a connection with rare inorganic nutrients such as aluminum or silica added with the humic acid; Söhngen (47) a connection with colloidal absorption and consequently greater availability of nitrogen and oxygen gases; Allen (1) with phosphate nutrition; Bortels (5) with molybdenum stimulation; Bottomley (6) and Mockeridge (34, 35) with "organic accessory food substance" or "auximone" nutrition; and Olsen (38), Remy and Rösing (39), Söhngen (47), Kaserer (25), Fred (19), and Koch et al. (28) with iron nutrition. Greaves (22) also suggested a protective action against poisons, such as arsenic, by colloidal absorption. It has often been suggested that humic acid increases the availability of one or more of the known constituents already present in the medium, rather than that it adds some stimulant or stimulants to the culture medium. At the outset of the present studies the writers were inclined to believe that probably some one of the numerous organic constituents of humic acid was specifically responsible for the stimulating effect.

It is the purpose of this paper to present evidence favoring the view that humic acid acts primarily by virtue of its iron component. The free living, aerobic soil organism *Azotobacter vinelandii* will receive chief consideration in respect of the studies on growth stimulation.

EXPERIMENTAL PROCEDURE

Preparation of humic acid

Humic acid is not a single homogeneous compound. It consists chemically of a large number of different compounds which may be extracted from soil organic matter or prepared synthetically from sugars, or other similar organic materials. In this paper humic acid will be considered as consisting of any of those compounds which may be *reversibly dissolved in dilute alkali and precipitated in dilute acid* (see also footnote 3). The chemical constitution

[1] A brief note relating to the work in this and the following paper appeared in *Science* (1931) 74: 522–4, and can be read simultaneously with advantage.

[2] Fertilizer and Fixed Nitrogen Investigations Unit, Bureau of Chemistry and Soils.

The writers are indebted to J. R. Adams, F. E. Allison, E. C. Shorey, O. Schreiner, and L. A. Pinck, for criticism and numerous suggestions.

TABLE 1

*Description of humic acids employed**

HUMIC ACID	SOURCE	DATE OF PREPARATION	NUMBER OF PURIFICATIONS§	METAL ADDED** PER 100 GM. SUGAR	DIGESTION	PER CENT Fe††
				gm.		*per cent*
(a) Natural						
I†	Soil A (garden)	VIII/–/29	4	.....	.....	(0.68)§§
II	Soil A	VII/–/30	4	.....	.....	0.68
III	Soil B (poor soil)	VIII/–/30	4‖	.....	.....	0.06‡‡
IV	Soil B	VIII/–/30	4	.....	.....	0.11
V	Soil C (rich soil	VIII/–/30	4	.....	.....	1.4
(b) Synthetic‡						
VI	50 g. glucose, 250 cc. 35 per cent HCl	X/–/30	4	.....	Boiling water bath for 2 hours	0.03‡‡
VII	50 g. glucose, 250 cc. 35 per cent HCl	X/–/30	6–10	Fe 1.0		0.30
VIII	50 g. glucose, 250 cc. 35 per cent HCl	II/–/31	6	.....	72° for 15 hours	0.03‡‡
IX	50 g. glucose, 250 cc. 35 per cent HCl	II/–/31	11	Fe 4.0		4.4
X	50 g. glucose, 250 cc. 35 per cent HCl	II/–/31	13	Fe 4.0		3.2
XI	50 g. glucose, 250 cc. 35 per cent HCl	II/–/31	2	Fe 4.0		4.4
XII¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	Mo 2.5		0.03‡‡
XIII¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	Al 2.5		0.03‡‡
XIV¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	P 2.5	Slowly boiled over flame for 15 minutes	0.03‡‡
XV¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	Si 2.5		0.03‡‡
XVI¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	Co 2.5		0.03‡‡
XVII¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	Mn 2.5		0.03‡‡
XVIII¶	50 g. glucose, 250 cc. 35 per cent HCl	V/–/31	5	.....		0.03‡‡
XIX¶	50 g. glucose, 250 cc. 35 per cent HCl¶¶	VII/–/31	4	.....		.....

* Throughout this paper the various humic acids will often be referred to in abbreviated form; thus Humic Acid I = H. A.-I, etc. Although the acids are employed in experiments in the form of their potassium salts, properties such as concentration and constitution will refer to the acids themselves.

† H. A.-I contained 4.3 per cent nitrogen as shown by Kjeldahl analysis. No other analyses of nitrogen in the different natural humic acids were made, but this analysis is probably representative.

‡ After the initial process of acid digestion the mixture, containing solid humic acid and charred sugar, was centrifuged (liquid discarded) and the humic acid in the precipitate extracted and purified as in the case of the natural humic acids.

§ As described in the text, each purification consisted of solution by alkali, reprecipitation with acid, centrifugation, and decantation.

¶ H. A.-XII to XIX were filtered instead of centrifuged.

‖ Washed with HCl for an unusually long time (16 hours), rather than about 1 hour, as in the case of the other natural humic acids.

** Added as a soluble salt at start of digestion, Fe as $Fe_2(SO_4)_3$, Mo as MoO_3, Al as $Al_2(SO_4)_3$, P as K_2HPO_4, Si as Na_2SiO_3, Co as $CoSO_4$, and Mn as $MnCl_2$.

†† Iron was analyzed by the colorimetric thiocyanate method of Scott (45), the sample of humic acid of known dry weight first being ignited and taken up in acid solution.

‡‡ The error with these relatively small quantities of iron (about 0.03 per cent) may be as great as ± 0.02 per cent.

§§ No iron analysis was made in the case of H. A.-I, but the latter was prepared in the same manner, and from the same soil, as H. A.-II, and was identical physiologically (see table 4D).

¶¶ The glucose employed to prepare H.A.-XIX was first treated to remove, so far as possible, the trace of iron impurity (0.00045 per cent) existing, by allowing a 20 per cent solution of glucose to stand for 1 hour at pH 10 and then filtering. Analyses indicated that a large part of the iron impurity in the glucose was removed by this treatment. Most of the iron present in H. A.-VI, VIII, and XII–XIX was undoubtedly derived from the HCl employed in their preparation.

of most of the individual organic compounds contained in humic acid is as yet unknown, although several of high molecular weight have been definitely identified (Schreiner and Shorey, 44, 44a, 44b, 46a, 46b). Natural soil humic acids contain generally nitrogen and various inorganic elements also. Thus Remy and Rosing (39) found that their carefully prepared active sample of soil humic acid contained 2.4 per cent nitrogen, 1.84 per cent Fe_2O_3, 3.12 per cent Al_2O_3, 6.9 per cent SiO_2, and 0.49 per cent P_2O_5. Russell (41, p. 165–178), Mattson (31), Waksman and Reusser (51), and Sven-Oden (50) present reviews of the present state of knowledge concerning the physical and organic chemistry of humic acid.

The natural humic acids used by the writers show an intensive growth-stimulative action with Azotobacter and Rhizobium, when these organisms are grown in artificial media. Remy and Rösing (39), Mockeridge (34), Olsen (38), and other writers have indicated that similarly prepared humic acids stimulate bacterial peptone decomposition, urea decomposition, alcoholic fermentation by yeasts, nitrification, denitrification, growth of legume nodule bacteria, and the growth of higher and lower green plants such as sunflower and duckweed, when grown under natural conditions.

A natural humic acid is prepared as follows. A 100-gm. sample of soil is washed twice with 100-cc. portions of *N* HCl, leached for 12 hours with 200 cc. 10 per cent KOH, and clarified by centrifugation. The decanted, centrifuged solution, containing the humic acid as soluble potassium humate, is reprecipitated by bringing the pH to 3.0–4.0 with HCl, and reclarifying by centrifugation. The precipitate, humic acid, is redissolved and reprecipitated generally about four times in all, and the final precipitate dissolved cautiously in aqueous KOH to give a slightly buffered solution at pH 7.5 containing about 10 mgm. humic acid and 0.4 mgm. nitrogen per cubic centimeter. It is then ready for use in physiological experiments, generally being added, with appropriate dilution, as 25–50 p.p.m. (= 25–50 mgm. per liter) calculated as humic acid. Azotobacter is unable to use either the carbon or the nitrogen in humic acid for nutrition (7, 8, 24, 29). The preparation of synthetic humic acids from glucose when different from that of natural ones, is described in table 1.

Cultural methods

The general methods of culture and analysis employed have been fully described elsewhere (7, 8, 9, 10) and will be but briefly reviewed here. Both the *Warburg* micro-respiration and *Erlenmeyer* flask techniques have been employed.

Warburg technique. The rate of oxygen consumption has been measured according to the physico-chemical, manometric, micro-methods worked out by Otto Warburg (52) for studying cell metabolism. The chief apparatus employed in connection therewith is illustrated in figure 1 and plate 1. The increase in rate of oxygen consumption with time gives a measure of the rate of growth, and is accompanied by a corresponding increase in the number of organisms, or, similarly, the dry weight, turbidity, and cell nitrogen. The manometers can be read frequently, and time curves of the increases of growth determined over very short periods of time, i.e., hours or minutes.

If desired, turbidimetric measurements of the actual growths in the *Warburg* vessels can be made at the end of an experiment, as a check on the indirect indications given by the respiration rate increases.

Unless otherwise indicated the temperature of the *Warburg* technique experiments has been 28 $\pm$0.01°C.

Erlenmeyer flask technique. The cultures have been grown chiefly in 25 cc. of liquid medium contained in 250-cc. *Erlenmeyer* flasks plugged with cotton. In preparing the flasks for sterilization, an additional 10 to 20 per cent by volume of distilled water has been added to make up for losses during sterilization. Sterilization has been performed in an autoclave maintained at 120°C. for 15 minutes. Inoculations have generally been made with one drop of a young culture, so that the inoculum constituted about 0.25 per cent by volume of the total culture. At times, also, the inoculum consisted of one drop of 1 cc. of culture diluted

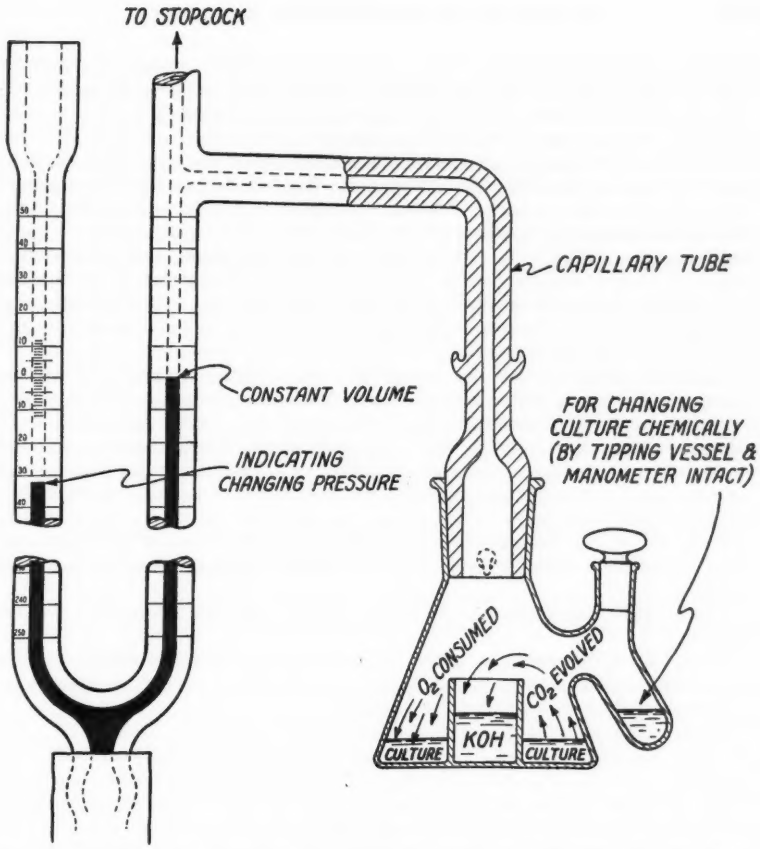

FIG. 1. DIAGRAM OF MANOMETER AND VESSEL EMPLOYED IN MEASURING OXYGEN CONSUMPTION AND GROWTH

The total volume of the vessel measured to the right-hand level of the manometric fluid (Brodie solution) is about 16 cc., 2 cc. of which is occupied by the culture and 0.30 cc. by the 2*N* KOH. The remaining volume is accounted for by a gas, usually air, at atmospheric pressure. The right-hand level of the manometric fluid is held constant; the CO_2 evolved in respiration is absorbed in the alkali; the oxygen consumption causes a slight decrease in internal pressure which is registered by a fall in the left-hand level of the manometric fluid; the reading thus obtained, when multiplied by a constant characteristic of the vessel, gives directly the number of cubic millimeters of oxygen consumed, with a usual accuracy of ± 0.5 cmm. The vessel constant varies from about 1 to 2, depending upon the temperature, volumes of gas and culture medium, the Bunsen absorption coefficient of the gas concerned, and the normal pressure of the manometric fluid. Under ordinary atmospheric conditions the amount of nitrogen consumption by *Azotobacter* is less than 1 per cent of the simultaneous oxygen consumption and may be neglected. If it is desired to change the chemical environment of the organism during the course of an experimental run, by addition of humic acid, nitrate, etc., a 0.10-cc. portion of an aqueous solution of the chemical made up to 20 times the desired concentration, and is placed in the side-cup initially and then tipped in when desired.

with 10 cc. sterile distilled water, yielding a culture inoculum 0.025 per cent that of the total culture. The cultures have been maintained in a large air incubator thermostat at 28 ±1°C. Care has been taken in each series of experiments to maintain constant all intended invariant conditions, such as volume, amount of inoculum, temperature, and particularly pH. The initial and final pH values of each culture have been determined colorimetrically, with one or more indicators and *La Motte* standards, and due allowance for pH differences has been made in interpretations of results. The rate of growth, as well as the rate of respiration, is slightly increased as the pH rises from 6.0 to 7.2. Ordinarily the experiments were run in duplicate or in triplicate. The cultures used to inoculate the *Erlenmeyer* flasks, as well as those used in the *Warburg* experiments, were grown in an air thermostat at 28 ±1°C. for 24 to 48 hours, in sterilized, aerated 250-cc. gas wash-bottles containing 50 to 100 cc. of culture medium. The aeration was obtained by means of compressed air passed through the bottles at the rate of about 100 cc. per minute.

Media of varying inorganic constitution have been employed. "Customary" medium contained the inorganic constituents remaining in the clear liquid obtained after the mixture "0.8 gm. K_2HPO_4, 0.2 gm. KH_2PO_4, 0.2 gm. $MgSO_4 \cdot 7H_2O$, 0.2 gm. NaCl, 0.1 gm. $CaSO_4 \cdot 2H_2O$, 0.01 gm. $Fe_2(SO_4)_3 \cdot 9H_2O$, 1,000 gm. H_2O" has been thoroughly shaken, allowed to stand and settle. The term "customary" concentration of salt or ion used throughout this paper will refer, however, to the concentration of that salt or ion obtaining in customary medium before the latter is allowed to settle. Chemical analysis shows that the clear solution contains about 15 per cent less phosphate, 40 per cent less calcium, and 99 per cent less iron than the unsettled solution. Unless otherwise stated, customary medium will always have been employed.

The inorganic constituents of "bicarbonate medium" consisted solely of 0.1 per cent $KHCO_3$.

When iron has been added in the form of citrate, tartrate, oxalate, or sulfate it has been reported in the tables upon the basis of 20 per cent composition, for simplicity, although the actual composition values range from 20–24 per cent.

When fixed nitrogen was present it was added at an initial concentration of 50 p.p.m. N, unless otherwise mentioned. This concentration of fixed nitrogen has been shown (7, 8) to prevent effectively all fixation of nitrogen in short time experiments of 2 days or less, and to place growth dependent entirely upon fixed nitrogen supply, even when nitrogen gas, as in air, is present. Recrystallized KNO_3 was used chiefly.

Although the inorganic constituents of the medium have been varied, the initial glucose concentration has been kept constant at 1 per cent in all experiments, unless otherwise mentioned, and during growth the concentration has rarely decreased below 0.2 to 0.5 per cent. The glucose used in these experiments (*Mallinckrodt, C. P.*) was reported by label to be quite pure, yielding, at a concentration of 1 per cent, a maximum total ash impurity in the cultures of 5 p.p.m. Actual analysis, made by a slightly modified thiocyanate method of Scott (45), showed that the iron fraction of the ash constituted only 0.045 p.p.m. This concentration of iron, however, was considerably larger, even, than that obtaining in clear settled glucose-free customary medium, namely, 0.012 p.p.m. Fe.

Growth has been measured turbidimetrically with a *Bausch and Lomb* nephelometer, after periods of incubation of ordinarily 36 to 60 hours. When the amounts of growth were very small, counting with a haemacytometer was also resorted to. In making the measurements it was always necessary to maintain uninoculated controls, since the amounts of precipitate were variable, and often quite appreciable. When appreciable amounts of turbidity occurred in the uninoculated controls, as would be caused by the presence of precipitated calcium phosphate, iron phosphate, etc., or when the pH of the inoculated culture was different from that of the control, it was often found expedient to acidify inoculated and uninoculated flasks to a constant pH of 3.5, i.e., just acid to brom-phenol blue, in order to dissolve the inorganic precipitates and to leave growth as the only cause of turbidity. Such acidification did not affect the turbidity caused by the organisms. The inoculum itself ordinarily did not change

the initial turbidity, but in some cases where growth was small or negligible, control corrections were necessary. At very high concentrations the color of humic acid necessitated a special correction in addition to that caused by the very slight turbidity of humic acid.

Growth has sometimes been measured in the *Erlenmeyer* technique by determining the final rate of respiration. Aliquot samples have been removed from the *Erlenmeyer* flasks and the respiration rates measured in *Warburg* vessels. This combined technique will be termed the "*Erlenmeyer-Warburg*" technique.

By "humic acid ratio" is meant the ratio of the final numbers, or likewise final respiration rates, turbidities, etc., of organisms in any given medium in the presence and absence of humic acid, or a substitute stimulator, after any given period of time, in our experiments usually 2 days but occasionally only a few hours or even a number of days. As will soon be evident, this ratio varies generally from 2 to 4, but may at times be either somewhat higher or lower, depending chiefly upon the *duration* of experiment, the concentration of humic acid, and the actual rate of growth in the absence of humic acid. The ratio will be calculated to two decimal points but no implication of this degree of accuracy shall be intended.

In comparing experiments performed by the *Warburg* and *Erlenmeyer* techniques it is to be borne in mind that in general the velocity of growth is greater in the case of the former because of agitation and other similar factors which permit of more constant and favorable conditions.

SUBSTITUTION OF NATURAL HUMIC ACIDS BY IRON COMPOUNDS AND SYNTHETICALLY PREPARED IRON-CONTAINING HUMIC ACIDS

Tables 2–8, inclusive, show that natural humic acids may be substituted by various organic or inorganic compounds of iron, such as ferric citrate, oxalate, tartrate, and sulfate, or metallic iron, under a wide variety of experimental conditions, as follow: (*a*) technique (*Warburg, Erlenmeyer, Erlenmeyer-Warburg*); (*b*) medium (customary and bicarbonate); (*c*) duration of experiment (18 hours to 6 days, including 25, 36, 40, 41, 48, 67, and 96 hours); (*d*) criterion of growth (turbidity, respiration rate, Kjeldahl-nitrogen content); (*i*) preparation of natural humic acids (Nos. I, II, V); (*f*) concentration of humic acids and iron compounds; (*g*) final pH (6.0–7.6); (*h*) age of inoculating culture; and (*i*) source of nitrogen [free N_2, and fixed nitrogen such as urea-N (500 and 200 p.p.m.) and NO_3-N (200 and 50 p.p.m.)]. Tables 10, 10A, 11, 11A, 13, and 14B also show humic acid substitution by various iron compounds and under an increased variety of experimental conditions.

Tables 9–15A, inclusive, show that synthetically prepared humic acid[3]

[3] The synthetic humic acids we have prepared (both the physiologically active and inactive ones) are quite similar to the natural humic acids in the important characterizing properties: (*a*) they are precipitated in mildly acid solutions (pH 3–5); (*b*) soluble alkali salts are easily formed; and (*c*) they are deeply colored, black, or dark brown (depending upon the concentration). In fact, we have observed no significant chemical differences. It is of no great interest here as to how exactly natural and synthetic humic acids are of the same chemical constitution, so long as, possessing many identical chemical properties, the same physiological functions are obtained. Eller and Koch (17) have prepared synthetic humic acids by various methods and have shown them to be similar to natural humic acids with respect to composition, solubility in water, alcohol, and ether, salt formation, color, and chemical reactions with Cl_2, HNO_3, Br_2, acetone, etc. Kawamura (27) has shown that the adsorption-concentration functions with respect to various bases are substantially identical for natural peat, and synthetic cane sugar, humic acids.

TABLE 2

Humic acid substitution by organic and inorganic iron compounds and non-substitution by various other metallic compounds (Erlenmeyer-Warburg technique)

SUBSTANCE ADDED	CONCENTRATION OF METAL	FINAL RESPIRATION RATE* (CMM. O_2 CC. HOUR) (RELATIVE GROWTH)	H. A.-RATIO
None	.....	62, 65, 67 / 56, 62, 59 } 62	....
H. A.-V (50 p.p.m.)	0.7 p.p.m. Fe	146, 167 } 157	2.53
Fe-citrate (10 p.p.m.)	2 p.p.m. Fe	100, 97 } 99	1.60
Fe-tartrate (10 p.p.m.)	2 p.p.m. Fe	103, 101 } 102	1.65
$Fe_2(SO_4)_3$	4 p.p.m. Fe	150	2.42
	3 p.p.m. Fe	187	3.02
	1 p.p.m. Fe	138	2.22
	0.6 p.p.m. Fe	107	1.73
	0.2 p.p.m. Fe	83	1.34
Fe-powder	10 p.p.m. Fe	91	1.47
	0.1 p.p.m. Fe	97	1.56
$CoSO_4$	10 p.p.m. Co	6	About 1.0 or less
	1 p.p.m. Co	68	About 1.0 or less
	0.1 p.p.m. Co	69	About 1.0 or less
$MnCl_2$	10 p.p.m. Mn	55	About 1.0 or less
	1 p.p.m. Mn	56	About 1.0 or less
	0.1 p.p.m. Mn	47	About 1.0 or less
$Al_2(SO_4)_3$	10 p.p.m. Al	66	About 1.0 or less
	1 p.p.m. Al	58	About 1.0 or less
	0.1 p.p.m. Al	65	About 1.0 or less
$NiCl_2$	10 p.p.m. Ni	0	About 1.0 or less
	1 p.p.m. Ni	40	About 1.0 or less
	0.1 p.p.m. Ni	79	About 1.0 or less
$ZnCl_2$	10 p.p.m. Zn	0	About 1.0 or less
	1 p.p.m. Zn	61	About 1.0 or less
	0.1 p.p.m. Zn	68	About 1.0 or less
$Cr_2(SO_4)_3$	10 p.p.m. Cr	19	About 1.0 or less
	1 p.p.m. Cr	54	About 1.0 or less
	0.1 p.p.m. Cr	53	About 1.0 or less
Na_2MoO_4	1 p.p.m. Mo	73	About 1.0 or less
	0.1 p.p.m. Mo	72	About 1.0 or less

Initial culture 1-day old *A. vinelandii*. Initial respiration rate 53 cmm. O_2/cc./hour. Analyzed after 40 hours. Flnal pH 6.8 ± 0.1 except 10 p.p.m. Al, Co, Ni, Zn, Cr, 6.4 ± 0.1. Source of nitrogen: N_2. V/19–21/31.

* The cultures were grown in *Erlenmeyer* flasks (28°C.) and the final relative growths measured as respiration rates (31°C.)

containing considerable fractions of iron was highly active physiologically, whereas when the iron fraction was negligible, or substituted by various other metals, as in tables 14, 14A, 14B, the physiological action was relatively

negligible. The latter observation confirms the general finding [Krzemieniewsky (29), Warmbold (53), Löhnis and Pillai (30), Remy and Rösing (39), and Mockeridge (34)] that synthetic humic acids prepared from sugars are physiologically inactive or substantially so, since, upon looking up the previous work it was observed that in every case iron was not employed in the preparations.[4]

TABLE 3

Humic acid substitution by ferric citrate and ferric sulfate (Erlenmeyer-Warburg technique)

	SUBSTANCE ADDED								
	None	H. A.-V		Fe-citrate			$Fe_2(SO_4)_3$		
Concentration (p.p.m.)	...	50	25	10	2	1	150	10	2
Concentration of Fe (p.p.m.)	...	0.7	0.35	2	0.4	0.2	30	2	0.4
Experiment 3:									
Final respiration rate*	173	484	.....	.....	528	.....	.....	368	.....
H. A.-ratio	...	2.80	.....	.....	3.06	.....	.....	2.13	.....
Experiment 3A:									
Source of N: N_2:									
Final respiration rate*	53	.....	73	.....	.....	78	.....	.....	70
H. A.-ratio	...	.....	1.38	.....	.....	1.47	.....	.....	1.32
Source of N: 200 p.p.m. urea-N:									
Final respiration rate*	227	.....	449	.....	.....	537	.....	.....	496
H. A.-ratio†	...	.....	1.98	.....	.....	2.36	.....	.....	2.18
Experiment 3B:									
Final respiration rate*	123	.....	246	196	.....	.....	160	.....	.....
H. A.-ratio	...	.....	2.0	1.59	.....	.....	1.30	.....	.....

Experiments 3; 3A; 3B: Initial culture 1-day; 5-hour; 1-day old *A. vinelandii.* Initial respiration rate 62; 20; about 2 cmm. O_2/cc./hr. Analyzed after 18; 48; 48 hours. Final pH 7.1 ± 0.3; N_2 6.8 ± 0.1, urea-N 6.3 ± 0.2; 6.6 ±0.1. Source of nitrogen: 500 p.p.m. urea-N; N_2 and urea; N_2. VI/3–4/31; V/11–13/31; V/23–25/31. 2 per cent glucose employed in experiment 3B (instead of 1 per cent).

* The cultures were grown in *Erlenmeyer* flasks (28°C., except Experiment 3, 31°C.) and the final relative growths measured as respiration rates (31°C.).

† It is probable that the much larger ratios obtained in urea-N (as compared to N_2) occur because of the very much greater growth and the consequently much greater need for iron.

[4] Blumenberg and Blumenberg (4) have, however, recently patented a process for making an organic iron compound suitable as a non-toxic plant stimulant, comprising treating comminuted cellulose material (sawdust, rice-hulls, ground corn-cobs, etc.) with an aqueous solution of iron sulfate and heating to about 120°C. until a dark brown or black color is assumed. We are unaware of any experimental work performed by Blumenberg and Blumenberg in connection with this patent, but its implications obviously receive strong scientific support from our work on the preparation of active synthetic humic acids and the greater physiological availability of organic iron compounds.

As shown in table 15A, the efficacy of a natural humic acid may be increased by increasing its iron content, just as in the case of a synthetic humic acid. The final amount of growth obtained increases with the iron added, regardless of from which humic acid the iron is derived.

TABLE 4

Humic acid substitution by organic and inorganic iron compounds (Warburg technique)

	SUBSTANCE ADDED									
	None	H. A.-II					Fe-citrate		Fe-tartrate	$Fe_2(SO_4)_3$
Concentration (p.p.m.)	...	125	50	40	32	16	50	32	50	10
Concentration of Fe (p.p.m.)	...	0.9	0.35	0.3	0.2	0.1	10	6	10	2
Experiment 4: Respiration rate 25th hour.	128	.....	.....	204	.....	.....	223	.....	234	232
H. A.-ratio	...	.....	.....	1.59	.....	.....	1.74	.....	1.83	1.81
Experiment 4A: Respiration rate 25th hour.	124	.....	153	.....	.....	.....	149	.....	.....	151
H. A.-ratio†	...	.....	1.24	.....	.....	.....	1.20	.....	.....	1.22
Experiment 4B: Respiration rate 25th hour	147	.....	323	.....	.....	.....	342	.....	.....	.....
H. A.-ratio	...	.....	2.20	.....	.....	.....	2.32	.....	.....	.....
Experiment 4C: Respiration rate 67th hour	137	287	.....	.....	212	179	.....	250	.....	.....
H. A.-ratio	...	2.10	.....	.....	1.55	1.31	.....	1.83	.....	.....

Experiments 4, 4A, 4B, 4C: Initial culture: 2-day old *A. vinelandii* diluted 3 times with customary medium containing 1 per cent glucose; 1½-day culture diluted 6 times with phosphate mixture containing 1 per cent glucose; 1½-day old culture diluted 6 times with customary medium containing 1 per cent glucose; 6-hour culture. Initial respiration rate (measured as cmm. O_2/cc./hr.): 10; 4; about 5; less than 1. Final pH 7.1 ±0.1; 7.1 ±0.1; 7.1 ±0.1; 6.8 ±0.1. Source of nitrogen: 50 p.p.m. NO_3-N; 50 p.p.m. NO_3-N; 50 p.p.m. NO_3-N; N_2. VIII/1–2/30; VIII/3–4/30; VIII/3–4/30; VIII/7–10/30.

† The H. A.-ratios are relatively low here because elements normally present in customary medium are relatively lacking.

Table 2 indicates that the growth stimulation effected by humic acid and by organic and inorganic iron compounds was not obtainable by use of certain other heavy metal compounds somewhat related to iron and sometimes suggested as being the cause of humic acid stimulation. The humic acid ratio, for the various concentrations of iron, 0.1 to 10 p.p.m., ranges from 1.34 at 0.02 p.p.m., to a maximum of 3.02 at 3 p.p.m., whereas the ratio for 0.1 to 10 p.p.m.

of the metals of the salts $CoSO_4$, $MnCl_2$, $Al_2(SO_4)_3$, $NiCl_2$, $ZnCl_2$, $Cr_2(SO_4)_3$, and Na_2MoO_4 is about 1.0 or less. Distinct toxicity was observed with 10 p.p.m. Co, 10 and 1 p.p.m. Ni, 10 p.p.m. Zn, and 10 p.p.m. Cr. In still

TABLE 4D

Humic acid substitution by organic and inorganic iron compounds (Warburg technique)

	SUBSTANCE ADDED									
	None	H. A.-I*	H. A.-II	Fe-citrate				Fe-tartrate	$Fe_2(SO_4)_3$	
Concentration (p.p.m.).....	..	40	40	100	50	20	5	50	30	10
Concentration of Fe (p.p.m.).....	..	0.3	0.3	20	10	4	1	10	6	2
Respiration rate 25th hour (cmm. O_2/cc. hour) (relative growth)...	48	159	159	186	158	146	145	174	161	156
H. A.-ratio.....	..	3.31	3.31	3.89	3.29	3.04	3.02	3.62	3.35	3.25

Two-day old *A. vinelandii* diluted 4 times with customary medium containing 1 per cent glucose. Initial respiration rate 12 cmm. O_2/cc./hour. Final pH 7.0 ±0.1. Source of nitrogen: 50 p.p.m. NO_3-N. VII/25–26/30.

* The sample of this humic acid was 1 year old, the other sample (H. A.-II) was freshly prepared.

TABLE 4E

Humic acid substitution by organic and inorganic iron compounds (Warburg technique)

	SUBSTANCE ADDED									
	None	H. A.-II		Fe-citrate					$Fe_2(SO_4)_3$	
Concentration (p.p.m.).....	...	12	6	50	25	12	6	3	10	5
Concentration of Fe (p.p.m.).....	...	0.08	0.04	10	5.0	2.5	1.2	0.6	2.0	1.0
Respiration rate 36th hour (cmm. O_2cc./hour) (relative growth)....	129	264	181	213	214	215	230	212	199	163
H. A.-ratio.....	...	2.05	1.40	1.65	1.66	1.67	1.78	1.64	1.54	1.26

One-half day old *A. vinelandii* undiluted. Initial respiration rate about 5 cmm. O_2/cc./-hour. Final pH 7.0 ±0.1, except 10 p.p.m. $Fe_2(SO_4)_3$, where 6.0 (Initial pH all 7.0). Source of nitrogen: N_2. VIII/11–12/30.

other experiments (*Warburg* technique) of short duration (4–6 hours) no beneficial effect was given by the following metals: Mo (as Na_2MoO_4)0.5, 5, and 50 p.p.m. of metal; Cu (as $CuSO_4$) 0.01, 0.5, and 2 p.p.m.; Ag (as Ag_2SO_4) 0.05, 0.5, and 5 p.p.m.; Al (as $l_2(SO_4)_3$), 0.05, 0.5, and 5 p.p.m.; and Mn (as

$MnCl_2$) 0.05, 0.5, and 5 p.p.m. Under these experimental conditions definite toxicity was observed with 1 p.p.m. of Cu, and complete toxicity in one-half hour with 2 p.p.m.; Al at 5 and 0.5 p.p.m. was definitely toxic, Mo at 50 p.p.m.

TABLE 5

Humic acid substitution by various concentrations of organic iron (Erlenmeyer technique)

	SUBSTANCE ADDED									
	None	Fe-citrate					H. A.-II			
Concentration (p.p.m.)..	....	0.2	1	3.2	8	25	1	2.5	8	25
Concentration of Fe (p.p.m.).....	....	0.04	0.20	0.64	1.6	5.0	0.007	0.017	0.054	0.17
Final gross turbidity....	21.8	28.6	36.6	42.7	48.5	50.8	31.4	56.2	86.8	100
Turbidity uninoculated controls.....	10.0	10.0	11.0	13.0	15.0	18.4	11.0	12.3	17.9	31.8
Final net turbidity (relative growth).....	11.8	18.6	25.6	29.7	33.5	32.4	20.4	43.9	68.9	68.2
H. A.-ratio.....	....	1.58	2.17	2.51	2.84	2.75	1.73	5.83	3.72	5.79

Inoculated with 1 drop 1-day old *A. vinelandii*. Analyzed after 48 hours. Final pH 6.8 ±0.1. Source of nitrogen: 45 p.p.m. NO_3-N. X/11–13/30.

TABLE 6

Humic acid substitution in relatively old cultures by various concentrations of inorganic iron (Erlenmeyer technique)

	SUBSTANCE ADDED									
	None	H. A.-II		$Fe_2(SO_4)_3$						
Concentration (p.p.m.)....	....	25	5	500	150	100	40	20	10
Concentration of Fe (p.p.m.).....	....	0.17	0.034	100	30	20	8	4	2
Experiment 6:									
Final net turbidity*.....	75	176†	115		256	158	122		94
H. A.-ratio.....	.....	2.35	1.54		3.42	2.11	1.63		1.26
Experiment 6A:									
Final relative Kjeldahl nitrogen H. A.-ratio...	1.00			1.30		2.44	3.00	3.04	2.30

Experiments 6, 6A: Inoculated with 2 drops 1-day old, 2 drops 1-day old *A. vinelandii*. Analyzed after 6 days, 6 days. Final pH 7.1 ±0.1, 6.8 ±0.1. Source of nitrogen: 200 p.p.m. NO_3-N, N_2. X/2–8/30, V/23–29/31.

* At 2 days the amount of growth in the presence of $Fe_2(SO_4)_3$ was indistinguishable from that in the control; the growth in the humic acid cultures was, of course, superior.

† It is probable, on the basis of other similar experiments, that 100 p.p.m. H. A.-II would have given a turbidity of 200–250, or a H. A.-ratio of 2.6–3.3.

very slightly toxic, and Ag and Mn were without either positive or negative effect at all concentrations studied. These *Warburg* technique experiments were conducted simultaneously with three different forms of nitrogen, urea-N (50 p.p.m.), NO_3-N (50 p.p.m.), and N_2, with identical results.

Table 8 and table 7, column 5, show that citrate, as sodium citrate, has no stimulative effect, *per se*, upon the growth of Azotobacter, under such circumstances that humic acid and ferric citrate give a large stimulation. The stimulative effect of ferric citrate is, therefore, to be referred directly to the iron fraction, the citrate fraction (or likewise tartrate, oxalate fraction, etc.) acting indirectly by holding the iron in a complex soluble form and tending to make it more soluble than inorganic iron.

TABLE 7

Humic acid substitution in bicarbonate medium by organic and inorganic iron compounds (Erlenmeyer technique)

	SUBSTANCE ADDED						
	None	H. A.-I	Fe-citrate	Na-citrate*	Fe-tartrate	Fe-oxalate	$Fe_2(SO_4)_3$
Concentration (p.p.m.)	....	25	25	25	25	25	10
Concentration of Fe (p.p.m.)	....	0.17	5	....	5	5	2
Final gross turbidity	15.6	104	75.5	13.1	67.3	72.0	37.9
	13.1	107	64.0	14.2	65.0	77.3	39.4
	11.4	98	....	15.6	....	79.5	....
Uninoculated control	10.5	8.5	9.3	11.4	6.6	13.3	32.8
Final average net turbidity 4 days (relative growth)	2.9	94	60.5	2.9	54.5	63.0	5.9
H. A.-ratio†	....	32.4	20.9	....	18.8	21.7	2.3

Inoculated with 1 drop 3-day old *A. vinelandii*. Analyzed after 4 days. Final pH 7.5 ±0.1. Source of nitrogen: 50 p.p.m. NO_3-N. VII/14–18–30.

* See also table 8.

† It is to be noted that the H. A.-ratios are exceptionally large in this experiment. These are occasionally obtained in bicarbonate medium and are due to a more pronounced lag phase in the controls. Probably by the fifth day the H. A.-ratios would have been approximately normal.

TABLE 7A

Humic acid substitution in bicarbonate medium by organic and inorganic iron compounds (Erlenmeyer technique)

	SUBSTANCE ADDED					
	None	H. A.-I	Fe-citrate	Fe-tartrate	Fe-oxalate	$Fe_2(SO_4)_3$
Concentration (p.p.m.)	....	25	25	25	25	10
Concentration of Fe (p.p.m.)	....	0.17	5	5	5	2
Final gross turbidity	42.5	80.4	44.7	116	82.9	73.5
Turbidity uninoculated controls	14.7	16.7	7.8	7.5	18.4	43.5
Final net turbidity 4 days (relative growth)	27.8	63.7	36.9	109	64.5	30
H. A.-ratio	....	2.29	1.33	3.92	2.32	1.08

Inoculated with 1 drop 1-day old *A. vinelandii*. Analyzed after 4 days. Final pH 7.4 ±0.1. Source of nitrogen: 50 p.p.m. NO_3-N. VIII/2–6/30.

TABLE 7B

Humic acid substitution in bicarbonate medium by organic and inorganic iron compounds (Erlenmeyer technique)

P.p.m. of $Fe_2(SO_4)_3$	.....	.....	....	....	....	....	....	....	....	....	....	10	5	1
P.p.m. of H. A.-I	100	25	12.5	2.5	0.25	....	....	....	....	....	....	....	....	....
P.p.m. of Fe-citrate	.....	.....	....	....	....	100	25	5	1	0.25	....	....	....	....
P.p.m. of Fe	0.68	0.17	0.08	0.02	0.002	20	5	1	0.2	0.05	....	2	1	0.2
Final gross turbidity	100	102	83.8	36.6	32.4	89.0	70.0	72.1	42.4	27.9	35.3	70.0	67.7	39.4
	101	98	93.0	35.9	29.6	83.8	80.6	79.5	.....	26.8	32.2	74.4	67.3	48.3
Turbidity uninoculated control	13.4	5.8	2.8	2.9	2.3	7.1	3.9	2.8	2.4	2.1	2.3	27.4	18.1	7.4
Final average, net turbidity (relative growth)	87.6	94.2	85.6	33.3	28.7	79.3	71.4	73.0	40.0	25.2	31.5	44.8	49.3	36.5
H. A. ratio	2.78	2.99	2.72	1.06	0.91	2.52	2.26	2.32	1.27	0.80	.....	1.42	1.57	1.16

Inoculated with 1 drop of 2-day old *A. vinelandii*. Analyzed after 48 hours. Final pH 7.5 ± 0.1. Source of nitrogen: 50 p.p.m. NO_3-N. VI/21–23/30.

Volumes of culture solution 34 cc., not 25 cc., as usual.

Sugar added after, not before, sterilization (as sterilized sugar).

In connection with tables 6A, 6, and 2, it is of interest that Elvehjem (18) has obtained similar inorganic iron stimulation with respect to baker's yeast, the humic acid ratios varying from 2.5 to 3.5. In one experiment, only 0.176 gm. dry weight per 200 cc. was obtained in a nutrient medium containing less than 0.025 p.p.m. Fe,[5] but 0.544 gm. (H. A.-ratio = 3.1) with 1 p.p.m. Fe, the duration of the experiment being 40 hours.

We have recently found (unpublished data) that under certain specific conditions (in brief, iron starvation in heavy cultures) the iron requirement of cultures grown in free nitrogen is from 2 to 10 times as great as that of cultures grown in fixed nitrogen. Under these conditions humic acid may be said to

TABLE 8

Humic acid substitution not provided by sodium citrate (Erlenmeyer technique)*

	SUBSTANCE ADDED			
	None	Na-citrate		H. A.-I
Concentration (p.p.m.)	....	25	100	25
Final relative turbidity	37.3	44.4	31.3	108
	33.9	44.8	36.2	111
	32.9	40.0	36.2	114
Uninoculated controls	4.4	8.9	8.1	11.7
	4.5	5.7	8.1	11.7
Final net turbidity (relative growth)	30.2	35.8	26.6	99.0
H. A.-ratio	....	1.18	0.88	2.26

Inoculated with 1 drop 2-day old *A. vinelandii*. Analyzed after 41 hours. Final pH 6.8 ±0.2. Source of nitrogen: N_2. V/22–24/30.

* See also table 7, column 5.

[5] Likewise, Krzemieniewsky (29) obtained very poor growths of Azotobacter in a medium containing no humic acid, but, here again, as in the case of Beijerinck's medium employed by Remy and Rösing (39) no iron was normally intentionally added. Ruhland (40) found that for the autotrophic hydrogen bacterium, *B. pycnoticus*, 0.07 p.p.m. Fe in the culture medium was necessary for normal growth, and that at 0.0005 p.p.m. growth and hydrogen consumption no longer took place. In our judgment, based upon a consideration of the literature (see especially "Preparation of Humic Acid" paragraph 2), many higher and lower plants grown in synthetic media will respond similarly, as in the case of Azotobacter and yeast, to treatment with various of the iron compounds considered in this paper. In other words, probably few plant media are optimal with respect to the form and concentration of iron supplied, whence the occurrence of growth stimulation by inorganic, organic, or humic acid iron, as the case may be. Students of Azotobacter physiology cannot fail to have observed the wide variation in amounts of nitrogen fixed per gram of sugar per 100 cc. medium, as reported by various investigators. A considerable number report consistent values of only 1 to 3 mgm., others report 10–15 mgm. We are confident that the variations often depend in great part upon the corresponding wide differences in concentrations of iron supplied either intentionally as inorganic iron (0–100 p.p.m.) or unintentionally as impurity in the sugar added. The iron supplied in the latter manner is an important factor in Azotobacter growth and is not so completely precipitated as the same concentration of purely inorganic iron, i.e., iron in sugar is partly in organic combination.

TABLE 9

The substitution of natural by synthetic humic acids (Erlenmeyer technique)

	SUBSTANCE ADDED																	
	None	H. A.-VII				H. A.-V				H. A.-IV			H. A.-III			H. A.-VI		
Description		Synthetic medium-Fe				Natural high-Fe				Natural medium-Fe			Natural medium-Fe			Synthetic low-Fe		
Concentration (p.p.m.)	..	100	25	10	5	100	25	10	1	100	25	10	100	25	10	100	25	10
Concentration of Fe (p.p.m.)	..	0.30	0.075	0.03	0.015	1.4	0.35	0.15	0.014	0.11	0.03	0.11	0.06	0.15	0.006	0.03	0.008	0.003
Final net turbidity (relative growth)	41	285	163	121	115	260	137	105	67	103	97	61	71	83	51	84	80	60
H. A.-ratio:																		
100 p.p.m.	..	6.95	.....	.....	.....	6.34	.....	.....	.....	2.51	.....	.....	1.73	.....	.....	2.05	.....	.....
25 p.p.m.	..	.....	3.98	.....	.....	.....	3.34	.....	.....	.....	2.36	.....	.....	2.02	.....	.....	1.95	.....
10 p.p.m.	..	.....	.....	2.95	.....	.....	.....	2.56	.....	.....	.....	1.49	.....	.....	1.24	.....	.....	1.46
1 or 5 p.p.m.	..	.....	.....	.....	2.80	.....	.....	.....	1.63	.....	.....	.....	.....	.....	.....	.....	.....	.....

Inoculated with 1 drop 2-day old *A. vinelandii*. Analyzed after 2 days. Final pH 6.6 ± 0.2. Source of nitrogen: N_2. XI/5–7/30.

TABLE 9A–B

The substitution of natural by synthetic humic acids containing iron (Erlenmeyer Technique)

	SUBSTANCE ADDED											
	None	H. A.-V			H. A.-VII			H. A.-IV			H. A.-VI	
Description		Natural high-Fe			Synthetic medium-Fe			Natural medium-Fe			Synthetic low-Fe	
Concentration (p.p.m.)	.....	250	50	18	250	50	18	250	50	18	250	50	18
Concentration of Fe (p.p.m.)	.....	3.5	0.70	0.25	0.75	0.15	0.05	0.27	0.05	0.02	0.10	0.02	0.005
Experiment 9B:													
Final net turbidity	25.3	.....	.....	46.8	.....	.....	48.8	.....	.....	40.0	.....	.....	22.0
H. A.-ratio	.....	.....	.....	1.85	.....	.....	1.93	.....	.....	1.58	.....	.....	0.87
Experiment 9A:													
Final net turbidity	285	658	557	.....	691	537	.....	581	475	.....	405	391	.....
H. A.-ratio	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
50 p.p.m.	.....	.....	1.95	.....	.....	1.89	.....	.....	1.67	.....	.....	1.37	.....
250 p.p.m.	.....	2.31	.....	.....	2.42	.....	.....	2.04	.....	.....	1.42	.....	.....

Experiments 9A, 9B: Inoculated with 1 drop 2-day old; 1 drop 2-day old *A. vinelandii*.
Analyzed after 11 days; 48 hours. Final pH 6.5 ± 0.1; 6.4 ± 0.1. Source of nitrogen: N_2; 200 p.p.m. NO_3-N. 1/17–28/31; XII/10–12/30.

TABLE 10

The substitution of natural by synthetic humic acids (Warburg technique)

	SUBSTANCE ADDED		
	None	H. A.-V	H. A.-VII
Description		Natural high-Fe	Synthetic medium-Fe
Concentration (p.p.m.)	...	25	50
Concentration of Fe (p.p.m.)	...	0.35	0.15
Experiment 1:*			
Respiration rate 14th hour (cmm. O_2/cc./hour) (relative growth)	80	100	105
H. A.-ratio	...	1.25	1.31
Experiment 2:†			
Respiration rate 34th hour	196	260	260
H. A.-ratio	...	1.33	1.33
Experiment 3‡:			
Respiration rate 34th hour	77	255	246
H. A.-ratio	...	3.31§	3.19

* Thirty-six-hour old *A. vinelandii* diluted 5 times with customary medium containing 1 per cent glucose. Initial respiration rate 10 cmm. O_2/cc./hour. Final pH 6.7 ±0.1. Source of nitrogen: N_2. I/12/31.

† Six-hour old *A. vinelandii*. Initial respiration rate about 2 cmm. O_2/cc./hour. Final pH 6.8 ±0.1. Source of nitrogen: N_2. I/13–14/31.

‡ Thirty-hour old *A. vinelandii* diluted 40 times with customary medium containing 1 per cent glucose. Initial respiration rate less than 1 cmm; O_2/cc./hour. Final pH 6.8 ±0.1. Source of nitrogen: N_2. I/13–14/31.

§ Fe-citrate (25 p.p.m. = 5 p.p.m. Fe) gave simultaneously a humic acid ratio of 3.41.

TABLE 10A

The substitution of natural by synthetic humic acids (Warburg technique)

	SUBSTANCE ADDED				
	None	H. A.-V	H. A.-VII	H. A.-VI	Fe-citrate
Description		Natural high-Fe	Synthetic medium-Fe	Synthetic low-Fe	
Concentration (p.p.m.)	....	25	50	50	5
Concentration of Fe (p.p.m.)	....	0.35	0.15	0.02	1.0
Final net turbidity 22nd hour (relative growth)	11.8	60.1	31.4	14.2	52.7
H. A.-ratio	....	5.10	2.66	1.20	4.47

Two-day old *A. vinelandii*. Final pH 6.2 ±0.2. Source of nitrogen: N_2. I/15/31. The cultures were grown in Warburg vessels and analyzed nephelometrically.

possess a specific effect on the chemical mechanism of nitrogen fixation, insofar as it provides iron. This iron may, however, be provided (as we have found) qualitatively as well by synthetic iron-containing humic acids, iron-organic

TABLE 11

The substitution of natural by synthetic humic acids (Erlenmeyer-Warburg technique)

	SUBSTANCE ADDED				
	None	H. A.-V	H. A.-IX	H. A.-X	Fe-citrate
Description		Natural high-Fe	Synthetic high-Fe	Synthetic high-Fe	
Concentration (p.p.m.)	....	10	10	10	10
Concentration of Fe (p.p.m.)	....	0.14	0.42	0.32	2
Final respiration rate* (cmm. O_2/cc./hour) (relative growth)	114	175	261	252	224
H. A.-ratio	....	1.54	2.29	2.22	1.97

Initial culture 2-day old *A. vinelandii* to which was added 1 per cent glucose and 0.1 per cent phosphate mixture. Initial respiration 94 cmm. O_2/cc./hour. Analyzed after 6 days. Final pH 6.7 ±0.1. Source of nitrogen: N_2. V/17–23/31.

* The cultures were grown in *Erlenmeyer* flasks (28°C). and the final relative growths measured as respiration rates (31°C.).

TABLE 11A

The substitution of natural by synthetic humic acids (Erlenmeyer-Warburg technique)

	SUBSTANCE ADDED								
	None	H. A.-V		H. A.-VIII		H. A.-X		Fe-citrate	$Fe_2(SO_4)_3$
Description		Natural high-Fe		Synthetic high-Fe		Synthetic low-Fe			
Concentration (p.p.m.)	...	5	25	5	25	5	25	1	2
Concentration of Fe (p.p.m.)	...	0.07	0.35	0.002	0.01	0.16	0.80	0.2	0.4
Experiment 1 (N_2): Respiration rate 18th hour (cmm. O_2/cc./hour) (relative growth)	61	103	122	67	90	93	103	65	65
H. A.-ratio	...	1.69	2.00	1.10	1.48	1.53	1.69	1.06	1.06
Experiment 2 (200 p.p.m. urea-N): Respiration rate 18th hour	163	186	187	160	168	200	220	209	232
H. A.-ratio	...	1.14	1.15	0.98	1.03	1.23	1.35	1.28	1.42
Experiment 3 (200 p.p.m. urea-N): Respiration rate 46th hour	236	.....	449	301	300	506	600	537	496
H. A.-ratio	...	.....	1.90	1.28	1.27	2.14	2.54	2.28	2.10
pH ±0.2 (experiment 3)	7.4	.....	6.8	7.4	7.4	6.8	6.2	6.2	6.1

Initial culture 5-hour old *A. vinelandii*. Initial respiration rate 20 cmm. O_2/cc./hour. Final pH 6.7 ±0.2, except where indicated in experiment 3. V/11–13/31.

TABLE 12

Comparison of the effects of different concentrations of iron-rich and iron-poor humic acids on growth (Erlenmeyer technique)

	SUBSTANCE ADDED															
	None	H. A.-V		H. A.-IX		H. A.-X		H. A.-XI		H. A.-VII		H. A.-VI		H. A.-VIII	
Description		Natural high-Fe		Synthetic high-Fe		Synthetic high-Fe		Synthetic high-Fe		Synthetic medium-Fe		Synthetic low-Fe		Synthetic low-Fe	
Concentration (p.p.m.)	...	10	60	10	60	10	60	10	60	10	60	10	60	10	60
Concentration of Fe (p.p.m.)	...	0.14	0.84	0.42	2.52	0.32	1.92	0.44	2.64	0.03	0.18	0.003	0.018	0.003	0.018
Final net turbidity (relative growth)	270	325	370	318	534	330	591	379	600	300	372	240	327	265	356
H. A.-ratio (10 p.p.m.)	...	1.20	....	1.18	.....	1.22	.....	1.40	.....	1.11	.....	0.89	.....	0.98	.....
H. A.-ratio (60 p.p.m.)	...	.....	1.37	.....	1.98	.....	2.19	.....	2.22	.....	1.38	.....	1.21	.....	1.32

Inoculated with 1 drop 1-day old *A. vinelandii*. Analyzed after 6 days. Final pH 6.7 ±0.1 Source of nitrogen: N_2. I/28–II/4/31.

TABLE 13

Comparison of effects of iron-rich and iron-poor humic acids on growth (Warburg technique)

	SUBSTANCE ADDED					
	None	H. A.-V			H. A.-X	H. A.-XIX
Description		Natural high-Fe			Synthetic high-Fe	Synthetic low-Fe
Experiment 1:*						
Concentration (p.p.m.)	..	225	75	25	225	225
Concentration of Fe (p.p.m.)	..	3.15	1.05	0.35	7.2	0.07
Respiration rate 25th hour (cmm. O_2/cc./hour) (relative growth)	95	350	276	199	200	116
H. A.-ratio	..	3.68	2.91	2.10	2.10	1.22

	SUBSTANCE ADDED						
	None	H. A.-V		H. A.-X		H. A.-XIX	
Description		Natural high-Fe		Synthetic high-Fe		Synthetic low-Fe	
Experiment 2:†							
Concentration (p.p.m.)	...	50	2	50	2	50	2
Concentration of Fe (p.p.m.)	...	0.70	0.03	1.6	0.06	0.015	0.0006
Respiration rate 12th hour	160	340	168	243	173	187	173
H. A.-ratio 50 p.p.m.	...	2.12	.....	1.52	.....	1.17	.....
H. A.-ratio 2 p.p.m.	...	.....	1.05	.....	1.08	.....	1.08

	SUBSTANCE ADDED					
	None	H. A.-V		H. A.-X	H. A.-VIII	Fe-citrate
Description		Natural high-Fe		Synthetic high-Fe	Synthetic low-Fe	
Experiment 3:‡						
Concentration (p.p.m.)	...	50	10	10	10	10
Concentration of Fe (p.p.m.)	...	0.70	0.14	0.32	0.003	2
Respiration rate, 13th hour	137	236	205	203	159	225
H. A.-ratio	...	1.72	1.50	1.48	1.16	1.64

* Initial culture 2-day old *A. vinelandii*. Initial respiration rate 60 cmm. O_2/cc./hour. Final pH 7.0 ±0.1. Source of nitrogen: N_2. VII/13–14/31.

† Initial culture 1-day old *A. vinelandii*. Initial respiration rate 60 cmm. O_2/cc./hour. Final pH 6.6 ±0.1. Source of nitrogen: N_2. VII/9/31.

‡ Initial culture 1-day old *A. vinelandii*. Initial respiration rate 30 cmm. O_2/cc./hour. Final pH 7.0 ±0.2. Source of nitrogen: N_2. V/8/31.

acids, etc., but is not supplied by iron-free humic acids. So far as concerns this paper, the findings provides strong additional proof that natural humic acid acts by virtue of its iron component, since humic acid definitely alleviates a specific case of proved iron-deficiency.

TABLE 14

Comparison of iron-humic acids with other metal-humic acids (Erlenmeyer technique)

		20 P.P.M. OF H. A.												
		V	VI	VII	IX	X	XI	XII	XIV	XV	XVI	XVII	XVIII	XIX
Description		Natural high-Fe	Synthetic low-Fe	Synthetic medium-Fe	Synthetic high-Fe	Synthetic high-Fe	Synthetic high-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe
Metal added in preparation of H. A.				Fe	Fe	Fe	Fe	Mo	P	Si	Co	Mn		
P.p.m. of Fe		0.28	0.006	0.06	0.84	0.64	0.87	0.006	0.006	0.006	0.006	0.006	0.006	0.006
Turbidity 42 hours (relative growth)	39.3*	100	44.6	67.6	75.7	80.6	161	46.9	52.9	58.0	53.2	56.5	46.1	42.0
Turbidity 66 hours	100													
	90.8	308	103	172	215	208	240	107	124	148	125	136	117	114
	87.2	292	109	174	215	240	224	109	136	162	140	130	111	110
Average	92.4	300	105	173	215	224	232	108	129	154	132	133	114	112
H.A.-ratio 42 hours	—	2.55	1.14	1.72	1.93	2.05	4.10	1.19	1.35	1.48	1.35	1.44	1.17	1.07
H.A.-ratio 66 hours	—	3.25	1.14	1.87	2.32	2.42	2.51	1.17	1.40	1.67	1.43	1.43	1.23	1.21

Inoculated with 4 drops 2-day-old *A. vinelandii*. Final pH 6.6 ±0.2. Source of nitrogen: N_2. VII/15–21/31.

* Average of three determinations 41.0, 38.9, and 37.9.

H. A.-XIII containing Al caused inhibition of growth.

TABLE 14A–B

Comparison of iron-humic acids with other metal-humic acids (Erlenmeyer-Warburg technique)

	HUMIC ACID											
		V	IX	X	XVI	XIV	XV	XVII	XII	XVIII	VI	Fe-citrate (10 p.p.m.)
Description of humic acid		Natural high-Fe	Synthetic high-Fe	Synthetic high-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	Synthetic low-Fe	
Metal added in preparation of H.A.			Fe	Fe	Co	P	Si	Mn	Mo			
Experiment 14A (25 p.p.m. H. A.):												
P.p.m. Fe	...	0.35	.....	0.80	0.008	0.008	0.008	0.008	0.008	0.008	0.008	.....
Final respiration rate*	123	246	.....	212	187	178	175	169	166	162	140	.....
H. A.-ratio	...	2.00	.....	1.72	1.52	1.45	1.42	1.37	1.35	1.32	1.14	.....
Per cent increase relative to H. A.-XVIII.	...	51.8	.....	30.8	15.4	9.9	8.0	4.3	2.5	.....	.....	.....
Experiment 14B (10 p.p.m. H. A):												
P.p.m. Fe	...	0.14	0.42	0.32	0.003	0.003	0.003	0.003	0.003	0.003	.....	2.0
Final respiration rate*	114	175	261	252	134	172	144	161	155	129	.....	224
H. A.-ratio	...	1.54	2.29	2.22	1.18	1.51	1.26	1.41	1.36	1.13	.....	1.97

Experiments 14A, 14B: Initial culture 1 cc. 1-day old *A. vinelandii* diluted 5 times with sterile customary medium; 2-day old *A. vinelandii* to which was added 1 per cent glucose and 0.1 per cent phosphate mixture. Initial respiration rate about 2 cmm. O_2/cc./hour; 94 cmm./cc./hour. Analyzed after 2, 6 days. Final pH 6.6 ±0.1; 6.7 ±0.1. Source of nitrogen: N_2; N_2. V/23–25/31; V/17–23/31. 2 per cent glucose in experiment 14A instead of 1 per cent.

* The cultures were grown in *Erlenmeyer* flasks (28°C.) and the final relative growths measured as respiration rates (31°C.) as cmm. O_2/cc./hour. H. A.-XIII, containing Al, caused inhibition of growth.

TABLE 15

The influence of duration of experiment upon the humic acid ratio with natural and synthetic humic acids (Erlenmeyer technique)

	SUBSTANCE ADDED (25 P.P.M.)								
	None			H. A.-V			H. A.-VII		
Description				Natural high-Fe			Synthetic medium-Fe		
Concentration of Fe (p.p.m.)	....	....	...	0.35	0.35	0.35	0.075	0.075	0.075
Duration (days)	2	3	5	2	3	5	2	3	5
Final net turbidity (relative growth)	10.0	68.1	160	18.6	100	219	23.4	117	243
H. A.-ratio	....	....	...	1.86	1.47	1.37	2.34	1.72	1.52

Inoculated with 1 drop 2-day old *A. vinelandii*. Analyzed after 2, 3, and 5 days. Final pH 6.3 ±0.3. Source of nitrogen: 200 p.p.m. NO_3-N. XII/10–15/30.

TABLE 15A

The improvement of natural humic acids by the addition of iron (Warburg technique)

	SUBSTANCE ADDED						
	None	H. A.-XX*		H. A.-XXI*		H. A.-XXII*	
Concentration (p.p.m.)	....	15	3	15	3	15	3
Concentration of Fe (p.p.m.)	....	0.06	0.01	0.15	0.03	0.15	0.03
Final relative turbidity 25th hour (H. A.-ratio)	1.00	1.65	1.01	2.10	1.26	2.06	1.13

Initial culture 1-day old *A. vinelandii*. Final pH, 6.8 ±0.1. Source of nitrogen: N_2. XI/6/31.

* H. A.-XX, a natural humic acid, was prepared from a garden soil (X/–/31), and contained a medium amount of iron, 0.4 per cent. H. A.-XXI and -XXII were prepared from H.A.-XX by allowing a 3.8 per cent solution of the latter containing 0.5 per cent $Fe_2(SO_4)\cdot 9H_2O$ in respectively (*a*) alkali (pH 9–10) and (*b*) acid (pH 3–4) to stand on the steam bath for 5 hours, at room temperature for 17 hours, and then repurifying eight times until the wash waters gave but traces of Fe H.A.-XXI and -XXII contained 1.1 ±0.1 per cent Fe, or an increase of 0.7 per cent over H.A.-XX.

THE HUMIC ACID RATIO AS A QUANTITATIVE FUNCTION OF THE IRON CONCENTRATIONS OF THE STIMULANT ADDED

Tables 16, 17, 18, and figures 2 and 3 indicate how far the qualitative humic acid substitution effects obtainable with the different iron compound types in tables 2–15 are capable of receiving quantitative functional expression and comparison. In making the comparisons, it is not the concentration of the compound, but the concentration of iron added by the compound which will be of interest. There are four different iron compound types: natural humic acid iron, synthetic humic acid iron, organic acid iron, and inorganic iron. Five compounds of the first type, 14 of the second, 3 of the third, and 2 of the fourth have been studied.

When all the experiments are considered as a whole, it is probable that, with the possible exception of inorganic iron, the other three types are approximately equivalent, within 50 per cent, in ordinary experiments lasting from one to

TABLE 16

The humic acid ratio as a function of concentration of iron in compounds of the organic acid iron type

DATA FROM TABLE NUMBER	Fe	HUMIC ACID RATIO		
		Fe-citrate	Fe-tartrate	Fe-oxalate
	p.p.m.			
2	2	1.60	1.65	.....
4	10	1.74	1.83	.....
4D	10	3.29	3.62	.....
7*	(5)	(20.9)	(18.8)	(21.7)
7A	5	1.33	3.92	2.32

* See footnote table 7.

TABLE 17

The humic acid ratio as a function of concentration of iron in synthetic humic acids

SYNTHETIC HUMIC ACID	NUMBER OF EXPERIMENTS	AVERAGE Fe CONCENTRATION	AVERAGE H. A.-RATIO
		(*p.p.m.*)	
XVIII (nothing)	4	0.006	1.21
XII (Mo)	4	0.006	1.27
XV (Si)	4	0.006	1.36
XVII (Mn)	4	0.006	1.41
XIV (P)	4	0.006	1.42
XVI (Co)	4	0.006	1.46
XIX (nothing)	4	0.007* (0.018)	1.11* (1.15)
VIII (nothing)	4	0.007	1.18
VI (nothing)	12	0.019	1.31
VII (Fe)	18	0.141	2.39
X (Fe)	14	0.40* (1.00)	1.60* (1.84)
IX (Fe)	5	0.59* (0.91)	2.00* (2.00)
XI (Fe)	3	0.72* (1.21)	2.67* (2.56)

* These average values do not contain a few certain exceptionally high-iron value experiments (0.07 per cent Fe with H. A.-XIX; 2.64 per cent with H. A.-XI; 2.52 per cent with H. A.-IX; 1.92, 1.6 and 7.2 per cent with H. A.-X) which would give misleading averages. The values in parentheses give the averages when all experiments are included. That the omitted iron values are exceptional and excessive can be seen from the fact that although the average iron concentration values are lowered considerably the average acid ratios are little affected. The same phenomenon will be observed in table 18 (except possibly with respect to inorganic iron, which discrepancy can be explained upon the basis of solubility relations).

several days, and when medium or small concentrations of stimulator are used. It is not to have been anticipated in tables 2–15 that the different iron com-

TABLE 18

The humic acid ratio as a function of concentration of iron in the four iron-compound types (based on tables 2–15 inclusive)

	TYPE 1	TYPE 2	TYPE 3	TYPE 4
Type of iron compound	Natural H. A.	Synthetic H. A.	Organic acid	Inorganic
Compounds included	I, II, III IV, V	VII, IX, X, XI	Fe citrate, oxalate, tartrate	$Fe_2(SO_4)_3$
Average using all experiments:*				
Average concentration of Fe	0.381	0.645	4.24	8.55
Average H. A. ratio	2.20	2.15	2.19	1.97
Number of experiments	70	45	39	30
Selected average:†				
Average concentration of Fe	0.268	0.309	0.271	0.386
Average H. A.-ratio	2.10	2.09	2.14	1.57
Number of experiments	66	40	11	7

* Except that in table 7 (see footnote to table 7).

† Based upon all iron concentrations of 1 p.p.m. Fe or less (see footnote to table 17).

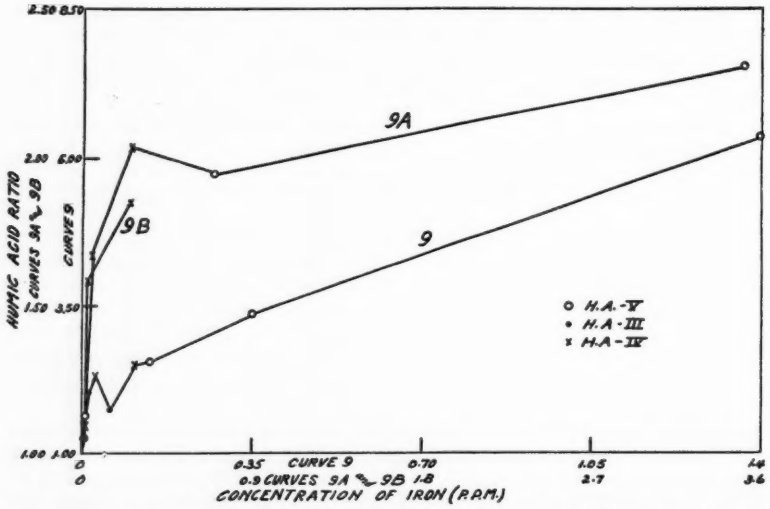

FIG. 2. THE HUMIC ACID RATIO AS A FUNCTION OF CONCENTRATION OF IRON SUPPLIED BY NATURAL HUMIC ACIDS

pounds should have all given exactly the same humic acid ratio as the control natural humic acid, either in any individual experiment, or as between different experiments. For one consideration, different natural humic acids give varying values among themselves, depending chiefly upon iron content, as shown in table 9 and figure 2. Humic acid ratios qualitatively representing

stimulation of the same order of magnitude are all that can be expected in any ordinary experiment, although it is undoubtedly possible to arrange experiments to yield substantially identical ratios by paying sufficient attention to certain secondary factors. Chief of these secondary factors determining the precise quantitative relations governing the value of the ratios are, as will be for the most part evident from inspection of the tables, (*a*) the duration of the experiment [see (11), "The Integral Functions"], (*b*) the concentration of the stimulant added (see tables 2, 4C, 4D, 4E, 5, 6, 6A, 7B, 9, 9A, 11A, 12, 13, and 15),

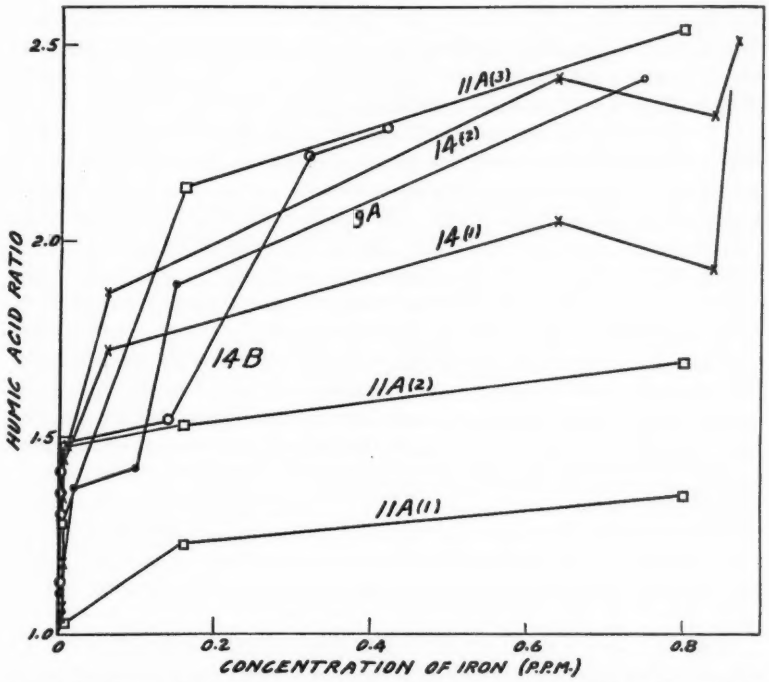

FIG. 3. THE HUMIC ACID RATIO AS A FUNCTION OF CONCENTRATION OF IRON SUPPLIED BY SYNTHETIC HUMIC ACIDS

(*c*) the chemical nature of the stimulant with respect to iron content, solubility, and physiological availability, and (*d*) the time value of the induction phase.

Table 16, which contains all the data available for direct comparison, shows that, at any given concentration of iron, ferric citrate, tartrate, and oxalate give the same humic acid ratio, within reasonable limits. In most of the experiments in table 16, the iron concentration is somewhat in excess of the minimum amount required to give approximately the optimum effect.

A comparison of figures 2 and 3 shows that about 0.3–0.8 p.p.m. Fe yields

about 60–80 per cent of the maximum humic acid ratio with both natural and synthetic humic acids. Curves 9, 9A, 9B, 11A, 14, 14B refer to tables 9, 9A, 9B, 11A, 14, 14B, respectively. To avoid confusion, in figure 3 we have not included all the available comparable data, but inspection of the excluded experiments (tables 9, 9B, 10A, 11, 12, 13, 14A) shows that they conform to those represented in the figure in indicating that, in general, in any given experiment, the humic acid ratio increases with the concentration of the iron added, independently of the particular synthetic humic acid employed. H. A.-VII is possibly a trifle more efficient per unit weight of iron than the other synthetic humic acids.

In table 17 are averaged the iron concentrations and respective humic acid ratios obtained in all experiments. An excellent general correlation is evident. H. A.-VII again appears to be somewhat the most efficient; but in comparing it to H. A.-IX and X, and possibly XI, one should remember that in the latter three cases considerably more iron has been employed (0.40, 0.59, and 0.72, instead of 0.14 per cent), probably more than the minimum necessary to give approximately the optimum effect. The superiority of H. A.-VII is, therefore somewhat more apparent than real. The *low*-iron synthetic humic acids containing Mo, Si, Mn, P, and Co appear to be very slightly better than those containing none of these elements (an average H. A.-ratio of 1.38 compared to 1.20); since they are all superior to about the same slight extent it is probable that these elements are not specific and that if the humic acids had been similarly prepared in the presence of more common salts, such as NaCl, $MgSO_4$, $CaSO_4$, the same slight superiority would have been obtained. At the same time we do not wish to preclude the possibility that stimulating, iron-free humic acids, containing the relatively rarer elements (Mn, Al, Co, Si, etc.) could not be prepared by some modified method. It is conceivable, for instance, that some organism being studied might have a special need for, say, aluminum, in which case a synthetic or natural humic acid containing aluminum impurity might very well act by virtue of supplying aluminum, instead of, or in addition to, iron.

Table 18 gives a comparison of the four types of iron compounds upon the basis of (*a*) all experiments, and (*b*) experiments with reasonable iron concentrations (optimal or sub-optimal, but not supra-optimal). The "Average Using All Experiments," is included as a matter of passing interest; it is quite obvious that here the H. A.-ratios of types 3 and 4 are incomparable to those of types 1 and 2 because the average iron concentrations involved are so much greater. The "Selected Average" excludes all iron concentrations above 1 p.p.m. Fe, amounting to 4 experiments (out of 70) in type 1; 5 experiments (out of 45) in type 2; 28 experiments (out of 39) in type 3; and 23 experiments (out of 30) in type 4 (see table 18, rows 6 and 9). The "Selected Average" iron concentrations are now of the same order of magnitude (0.266 to 0.386 p.p.m.) and the humic acid ratios also approximately so. The inorganic iron appears to be slightly less effective than the other three types, but it must be

remembered that 0.386 p.p.m. inorganic iron would be on the average only 25–50 per cent soluble, and, when this allowance is made, the ratio of 1.57 as compared to 2.10, 2.09, and 2.14, is quite within expectation based upon concentration functions similar to those represented in figures 2 and 3.

The general conclusion that a given *sub-maximal* amount of *soluble* iron, viz., about 0.2–1.0 p.p.m. or less, gives, independently of iron-compound type, the same order of magnitude of humic acid ratio, is subject to the provision that the experiments be allowed to proceed long enough to avoid any limitation through induction, viz., 36–48 hours or more, and to consume the physiologically active iron added. Consideration of the induction period is essential at this point in order to prevent any misinterpretation of the tables regarding the exact equivalence of natural humic acids and iron compounds, especially inorganic iron. In this connection, tables 3B, 7, 7A, 7B, and 11A (exp. 1), give some indication that $Fe_2(SO_4)_3$, especially, is not exactly equivalent to ferric citrate[6] or humic acid with respect to certain time relations. The lengths of

[6] Olsen (38) has recently indicated in a preliminary publication that humic acid may be quantitatively replaced by ferric citrate in stimulating plant growth, the experiments having been conducted with *Lemna polyrhiza* and *Helianthus annuus*. His humic acid consisted of equal parts of aqueous alkaline extract of bacterized peat and ammoniacal extracts of peat. With the former plant 183 mgm. of dry matter were obtained without humic acid, and 452 mgm. with 15 p.p.m. of humic acid (10 p.p.m. $FeCl_3$ being present in both cases, in an inorganic medium made up with distilled water); whereas, when the $FeCl_3$ was replaced with one-half the equivalent quantity of Fe-citrate (but without humic acid being present, 476 mgm. of dry matter were likewise obtained. The same results with respect to equivalence of Fe-citrate and humic acid were obtained when tap water was used instead of distilled water; moreover, Fe-citrate and humic acid together gave no higher yields than either alone, within 10 per cent; the pH in the aforementioned experiments was maintained at approximately 7.5, at which pH iron is more or less completely precipitated in the absence of organic matter. Similar results were obtained with Helianthus grown at pH 5.2 to 6.8. Saeger (42) likewise obtained stimulation effects with Spirodela and Lemna, using organic extracts derived from either a 1 per cent $NaHCO_3$ extract of peat, or a watery extract of autolyzed yeast.

In view of the apparently clear-cut nature of Olsen's experiments, it is pertinent to note that several important criticisms obtain. In the most decisive experiment, with Lemna, there were only eight experimental cultures reported, in six of which the amounts of dry matter obtained were identical within experimental error; in other words, the chief conclusion of the paper is based upon the behavior of virtually two divergent cultures. A more specific criticism, however, is that no experiments were performed in which the humic acid was added in the absence of added Fe, so that there remains the uncertainty as to (*a*) whether the growth would have been as great in the presence of humic acid and the absence of $FeCl_3$, as in the presence of both; or (*b*) conversely, whether the humic acid actually increased the availability of the $FeCl_3$ added. Also, there were no experiments where citrate was added in the absence of Fe; i.e., the effect of the Fe-citrate may have been due to the citrate entirely, so far as the experiments were capable of indicating. Finally, the experiments were not conducted under sterile conditions with respect to organisms other than Lemna; according to Clark (13, 15), under sterile conditions, the growth of Lemna is not stimulated by organic extracts.

Probably the most extensive work connecting the humic acid effect with addition of contained iron, previous to that being reported, is that of Remy and Rösing (39). These authors showed that (*a*), as the humic acid was freed from iron by chemical purification, its activity rapidly decreased to zero in solutions to which no inorganic iron had been added purposely;

the induction periods, vary from a few hours to a day or more (11, "Induction in Humic Acid Substitution Compounds"). It is obvious, therefore that in the early stages of growth, especially during the first day, those substances with the shortest induction period will for a time appear superior, often considerably so.

IS HUMIC ACID STIMULATION DUE SOLELY TO IRON?

It is true that not only do humic acid and iron compounds cause similar growth stimulations, but also, *where humic acid has no stimulative effect, iron compounds likewise have no stimulative effect.* This is shown in tables 19, 19A, 19B, and 20, 20A for Azotobacter and Chlorella, respectively, and is true even though at the same time changing other chemical substances and physiological conditions actually can produce stimulation, e.g., Azotobacter: increasing the temperature to 33-35°C., lowering the oxygen concentration to 0.05 atm., adding urea-nitrogen; Chlorella: increasing the illumination, by growing the cultures under non-stagnant conditions (i.e., with agitation by rapid aeration), by employing much more dilute inoculations, and to a limited extent, by changing the concentrations of some of the chemical constituents of the medium.

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this was shown also by Krzemieniewski (29) and Mockeridge (34); and (*b*) upon the addition of inorganic iron to a medium containing inactive humic acid the growth of Azotobacter became as great as in the presence of active humic acid. Thus it is seen that they claimed that humic acid not only added contained iron, but could, under certain circumstances, increase the availability of inorganic iron added to the medium. Unfortunately several important control experiments were neglected. With respect to (*b*) it was not shown in the same series of experiments (No. 152–159) that the inorganic iron (Fe_2O_3) added would not have given as large an effect even in the absence of inactive humic acid, yet other experiments (viz., No. 121, compared to Nos. 109 and 110) indicated that this would have been substantially the case. It was not shown that iron plus active humic acid gave results equal to, but not greater than, iron alone, to prove that the action of the active humic acid was confined to that of supplying iron. With respect to (*a*), they were unable to answer satisfactorily the objection that at the same time that the humic acid was being freed from iron it was (as they showed) likewise being freed from aluminum, phosphorus, and silica, and to about the same proportional extent, so their results really indicated only that humic acid lost its effect as something was removed or destroyed by the process of purification. The criticisms of the work of Remy and Rösing, and likewise that of Olsen's work, are advanced not with the purpose of throwing doubt upon the chief final conclusions arrived at, but with the purpose of indicating the insecurity, or rather, incompleteness of the data used in reaching the conclusions. One general criticism, perhaps the most important of any, must never be lost slight of in connection with all the work on humic acid, whether in connection with iron or other substances, by workers prior to about 1920, namely, that no pains were taken either to regulate or to observe pH changes. With Azotobacter, growth of which ceases at pH 6, and greatly decreases above pH 8, this is essential. The media employed by the earlier workers generally contained but little phosphate buffer (0.2 to 0.5 gm. potassium phosphate per liter), and with the high amounts of iron generally employed (even 100 to p.p.m.) it was inevitable that pH changes over wide ranges should occur. In attempting to interpret the earlier work consideration of the effect of pH cannot be too strongly emphasized. The report given in this footnote of the work of Olsen and of Remy and Rösing, together with our work reported in this paper, will give the reader a summarizing account of the chief *experimental* work on the relation between iron and the physiologically beneficial action of humic acid.

TABLE 19

*The absence of stimulation upon addition of humic acid or iron compounds to a very young culture of Azotobacter**

SUBSTANCE ADDED	CONCENTRATION	CONCENTRATION Fe	EXPERIMENT 19		EXPERIMENT 19A		EXPERIMENT 19B	
			Final net turbidity 24th hr.	H. A.-ratio	Final respiration rate 10th hour	H. A.-ratio	Final respiration rate 20th hour†	H. A.-ratio
	p.p.m.	*p.p.m.*						
None	.....	....	41.4	....	98	....	6.8	.
H. A.-I	40	0.27	....	....	91	0.93	...	.
	25	0.17	40.7	0.99	..	....	...	.
H. A.-II	250	1.7	....	....	..	....	5.8	1
	125	0.85	....	....	..	....	5.9	1
	62	0.43	....	....	..	....	5.4	1
	31	0.21	....	....	..	....	5.3	1
	40	0.27	....	....	98	1.00	...	.
$Fe_2(SO_4)_3$	30	6	....	....	95	0.97	...	.
	10	2	....	....	93	0.95	...	.
Fe-tartrate	50	10			92	0.94		
Fe-citrate	100	20	....	....	96	0.98	...	.
	50	10	....	....	95	0.97	...	.
	20	4	....	....	90	0.92	...	.
	5	1	....	....	98	1.00	...	.
	31	6	....	....	..	....	6.0	1
	25	5	39.4	0.95	..	....	...	.
	5	1	46.4	1.12	..	....	...	.
	1	0.2	47.7	1.15	..	....	...	.
	0.25	0.05	46.2	1.12	..	....	...	.
Fe-citrate plus 25 p.p.m. H. A.-I (0.17 p.p.m. Fe)	100	20	42.6	1.03	..	....	...	.
	5	1.2	45.7	1.11	..	....	...	.
	1	0.37	41.1	1.00	..	....	...	.
	0.25	0.22	43.1	1.04	..	....	...	.

Experiments 19; 19A; 19B: Technique *Erlenmeyer; Warburg; Warburg.* Initial culture 1 drop 1-day; 1-day; 6-hour old *A. vinelandii.* Initial respiration rate—; 15; about 0.1 cmm. O_2/cc./hour. Final pH 7.0 ±0.1; 7.1 ±0.1; 6.8 ±0.1. Source of nitrogen: N_2; 50 p.p.m. NO_3-N; N_2. VI/26–27/30; VII/18/30; VIII/7–10/30.

* For explanation of this phenomenon see 11, "length of the induction period." It has been found empirically that in general no humic acid stimulation is observed until the cultures have attained a heaviness of growth corresponding to a respiration rate of about 5 or more cubic millimeters O_2 per cubic centimeter per hour.

† Accurate to ± 1 cmm.

We do not wish to be understood as precluding the possibility of humic acid stimulation of pure cultures of Chlorella under conditions other than we have employed, even though Clark (13, 15) also obtains stimulation of Lemna only in non-sterile medium but not in sterile medium. The universally obtained

TABLE 20

The absence of stimulation of growth of pure cultures of Chlorella by humic acid or iron compounds under specific conditions

		EXPERIMENT 20		EXPERIMENT 20A
Duration (hours)		142		118
Initial concentration cells (millions per cubic centimeter)		0.066*		0.23*
Final pH		6.9		6.7
Substance added	Concentration	Final dry weight† (mgm. dry matter per 100 cc.) (absolute growth)	Final net turbidity (relative growth)	Final net turbidity (relative growth)
	p.p.m.			
None	.....	60.0	100 ± 5	100 ± 15
Citric acid (neutralized to pH 7)	250	66.7	100 ± 5	.....
	100	....	100 ± 5	.....
	25	....	100 ± 5	100 ± 15
	12.5	....	100 ± 5	.....
	2.5	....	100 ± 5	.....
	0.5	....	100 ± 5	.....
Fe-citrate	100	60.0	100 ± 5	.....
	25	....	100 ± 5	100 ± 15
	12.5	....	100 ± 5	.....
	2.5	....	100 ± 5	.....
	0.5	....	100 ± 5	.....
$Fe_2(SO_4)_3$	20	65.2	100 ± 5	.....
	5	....	100 ± 5	100 ± 15
	2.5	....	100 ± 5	.....
	0.5	....	100 ± 5	.....
H. A.-I	100	62.3	80 ± 5	100 ± 15
	25	....	100 ± 5	100 ± 15
	12.5	....	100 ± 5	.....
	2.5	....	100 ± 5	100 ± 15
$Fe_2(SO_4)_3$ plus H. A.-I	5	....	.....	.....
	25	....	.....	100 ± 15
$Fe_2(SO_4)_3$ plus neutralized citric acid	5	....	.....	.....
	25	....	.....	100 ± 15

* The final concentration of cells (determined with a haemacytometer) was about 6 million per cubic centimeter, in all cases in both experiments, corresponding to a generation time of about 25 hours.

† The dry weight measurements represent a composite determination, in the case of any given compound, of all the respective concentrations, and were made by combining 25-cc. portions from each flask of each concentration of a given compound and centrifuging the cells, transferring the cell precipitates to small weighed centrifuge cups, recentrifuging, decanting the clear liquid, and reweighing after drying *in vacuo* at room temperature. The variability of only 5 per cent confirms the turbidity measurements.

The pure cultures of Chlorella were grown under much the same conditions employed by Warburg (52). The medium consisted of 1,000 cc. of H_2O containing 2.46 gm. $MgSO_4 \cdot 7H_2O$, 1.22 gm. KH_2PO_4, 1.26 gm. KNO_3, and 0.0042 gm. $Fe_2(SO_4)_3 \cdot 9H_2O$. Under the conditions employed growth was unaffected by the presence or absence of $CaCO_3$. 50 cc. sterile medium were contained in each 500-cc. rubber-stoppered flask. The flasks were aerated in series with washed air containing 5 per cent CO_2 (the optimum CO_2-content), and were vertically illuminated at a distance of 34 inches by 100-watt Mazda frosted lamps so arranged that substantially uniform illumination was obtained. The temperature was 23–25°C. Cultures were run in duplicate.

stimulation of growth of impure cultures of the lower green plants by organic extracts probably takes place, or is induced, as a result of the relatively more rapid depletion of *soluble* iron from the medium caused by bacteria or other similar contaminating forms, whose iron requirements are presumably higher. Consequently, if stimulation of pure cultures is desired, care must be taken to eliminate iron from the culture medium or that already present as impurity will generally suffice to prevent stimulation by iron bound with organic matter intentionally added. Cultures with low iron requirements, as compared to those with high requirements, can undoubtedly be relatively more easily accommodated by the slowly and very slightly soluble, inorganic iron. It is probable that pure cultures grown for several transfers in a medium as free from iron as possible would submit to stimulation.

TABLE 21

The influence of Ca-humate upon the growth of Azotobacter in a calcium-deficient medium (Erlenmeyer technique)

	CALCIUM FREE* CUSTOMARY MEDIUM PLUS SOLUBLE Ca AS						
	Nothing	$CaSO_4 \cdot 2H_2O$	Ca-humate†				H. A.-VII (200 p.p.m.) (no Ca)
Concentration of soluble calcium added (p.p.m.)	.	15	2.4	9.4	24	48	..
Final count (millions per cubic centimeters)	6	50	25	35	45	50	8
Per cent growth increase	.	730	315	480	650	730	33

Inoculated with 1 drop of 5-hour old *A. vinelandii*. Analyzed after 4 days. Final pH 6.9 ±0.1. Source of nitrogen: N_2.

* Prepared by omitting the customary 100 p.p.m. $CaSO_4 \cdot 2H_2O$.

† A synthetic preparation containing 4.7 per cent Ca and less than 0.03 per cent Fe. In the presence of 100 p.p.m. $CaSO_4 \cdot 2H_2O$ this iron-poor Ca-humate preparation has substantially no stimulative action.

It is realized that various humic acids, prepared from various soils or sugars under various conditions, might conceivably exert their stimulating effects from various causes, i.e., from contained iron, contained aluminum, contained silica. As shown in table 21, when some essential element, in this instance calcium, is omitted from the culture medium of Azotobacter, then humic acid containing that element may supply it in a form which is comparable although not superior in availability to the form ordinarily supplied. As a matter of fact, indeed, because of the high molecular weight of humic acid, the Ca-humate (4.7 per cent Ca) is less effective per unit dry weight of humic acid than $CaSO_4 \cdot 2H_2O$. The H. A.-VII culture acts as a control on the Ca-humate cultures in showing that a calcium-free synthetic iron-containing humic acid can not replace an iron-free synthetic calcium-containing humic acid in a calcium-free medium. All that can be reasonably claimed for the data of this paper is that contained iron is probably the chief cause of humic acid growth stimulation.

DISCUSSION

Our chief endeavor has been to prove or to establish, so far as possible, the chemical nature of humic acid stimulation, rather than to indicate a few suggestive and limited experiments in connection therewith. We have therefore not hesitated to present in this paper and in the following paper (11) a comprehensive body of experimental material. It should easily be evident that this procedure is essential, because of the manifold factors which must be studied before any satisfactory conclusion or conclusions can be reached concerning the cause or mode of operation of humic acid stimulation. We cannot emphasize too strongly this general consideration.

It is obviously impossible, in this long report, to give more than an indication of the broader implications of these findings and to make reference to the most pertinent recent literature (2, 3, 4, 6, 12, 13, 14, 15, 16, 18, 19, 20, 21, 23, 24, 32, 33, 34, 35, 36, 37, 38, 41, 43, 44b, 46, 49, 51, 54, 56, and works cited therein). It is believed that these findings relate to various fields outside the immediate scope of this paper, as follow: The chemical constitution of the active components of the numerous growth-promoters or essential nutrients such as yeast-water, plant extract, peat extract, soil extract, bios, auximones, egg albumen, egg extract, Rhizopin, and vitamin B; the substitution of certain of these substances of unknown general chemical constitution by synthetic iron-containing humic acids, in the preparation of growth media; the iron nutrition of higher green plants grown in water cultures; the influence of soil humus and organic matter upon soil fertility, as a function of iron supply. It would be desirable to study plants with high requirements for certain less common elements, such as Al, Mn, Cu, or B, and determine whether these could be supplied especially readily in the form of humates. In regard to iron humates, further work would be desirable in preparing natural or synthetic humic acids with in general still shorter induction periods, or greater effectiveness per unit of dry weight of humic acid (i.e., with still higher iron contents). This could be done by separating humic acids into each of their various organic acid constituents and then selecting, if there were any differentiation, the most superior, especially those compounds of low molecular weight. Finally, there remains the fundamental question of why humic acid iron is in general so much more available than the other types, at least those so far studied; at present we incline to the view that the explanation lies in the adsorbed condition of the humic acid iron (27) and its high solubility. In this connection study might be made of other colloidal carriers of iron besides the humic acids. All these problems yet to be solved should not be confused with the chief problem brought to solution in this paper, namely, the establishing of the rôle of iron in the stimulation of growth by humic acid.

SUMMARY

Upon the basis of a number of independent lines of evidence it is shown that humic acid causes stimulation of Azotobacter growth primarily by virtue

of its *iron* component; other inorganic impurities and the organic fraction are substantially inactive.

Natural humic acid may be substituted for more or less satisfactorily by (*a*) complex, non-ionized organic acid iron such as ferric citrate, tartrate, oxalate; (*b*) inorganic iron such as ferric sulfate, metallic iron; (*c*) humic acids prepared synthetically from glucose and ferric sulfate, and containing considerable quantities of iron.

Natural humic acid was not substituted by (*a*) humic acids prepared synthetically from glucose to contain no iron, or as little iron as possible; (*b*) iron-free synthetic humic acids containing various metals such as Al, Co, Mn, Si, Mo, and P; (*c*) salts of such elements as Al, Co, Mn, Mo, Ni, Zn, Cr, Cu, and Ag at various metal concentrations ranging from 0.01 to 50 p.p.m.

The activity of a natural humic acid, per unit of dry weight, may be increased by synthetically increasing its iron content.

Natural and synthetic iron-containing humic acids have been shown to alleviate a specific case of iron-deficiency occurring under certain conditions of nitrogen fixation.

Under conditions where humic acid exerted no stimulative effect the iron substitution compounds likewise exerted no effect, even though other chemical substances and physiological conditions could at the same time increase the growth rate considerably.

A statistical study of all the available data shows that, from a quantitative standpoint, *a given sub-optimal amount of soluble iron (about 0.2 to 1 p.p.m. or less) gives the same order of magnitude of increased growth, independently of whether the iron is added as contained in natural humic acid or in any of its substitution compounds.* This statement requires the qualification that the experiments must proceed long enough to avoid limitation through induction period differences and to allow complete consumption of the sub-optimal physiologically active iron added. In the case of Azotobacter, natural humic acids commence to exert stimulation some hours sooner than organic acid iron compounds, and the latter again somewhat sooner than inorganic iron compounds; the synthetic iron-containing humic acids behave substantially as the natural ones. Consequently, *in experiments of relatively short duration it will often be found that inorganic iron, and even organic acid iron, will provide considerably less satisfactory stimulation than iron contained in natural or synthetic humic acids.*

In view of this work, humic acid is to be classed as both a nutrient which provides iron for growth and nutrition, and a stimulant which provides iron in a form in general *more highly available* than that usually supplied in the medium. Humic acid is not a plant-vitamin, or any other constituent classifiable as *essential* to the medium, since it may be replaced by other substances containing iron.

Like many other inorganic and organic compounds of calcium, synthetically prepared calcium humate may supply calcium to Azotobacter in a calcium-free

medium, but in a form *no more available* than that usually supplied in the medium (calcium sulfate). Other similarly incomplete media could undoubtedly be improved in a similar manner by humates containing the respective lacking element, but this action would be a matter simply of nutrition and not of increased availability, and relatively large concentrations of the humates would have to be employed.

Strictly interpreted, the reported findings apply only with respect to Azotobacter; but many reasons obtain for believing them to be of widespread application. Even though humic acid might with certain organisms and under certain special circumstances, play some rôle other than here assigned, the fact that in any case it is capable of increasing the availablity of iron would seem established beyond question. Humic acid is probably one of the few growth-promotors whose determining constitution has been thus established.

Upon the basis of this work explanations in part are offered for (*a*) the widely different amounts of nitrogen fixed by Azotobacter per gram of sugar consumed, when time is not a variable; (*b*) Clark's finding that growth stimulation of certain lower green plants by organic extracts occurs only in the presence of contaminating organisms; (*c*) the nature of the chemical constitution of various growth-promoters; and (*d*) the beneficial influence of humus and organic matter upon soil fertility.

Postscript added to galley proof. Since submission of the manuscript of this paper to press, Dr. Earl S. Johnston, division of radiation and organisms, Smithsonian Institution, Washington, D. C., has very kindly performed some experiments on the effect of natural and synthetic humic acids upon the growth of tomato plants in water cultures under artificially maintained conditions of temperature, humidity, and lighting, and also under greenhouse conditions. The following findings appear reasonably certain. A single initial dosage of 0.5 to 5 p.p.m. iron as iron humate provides iron in a form equal to, or superior to, dosages of 1 to 5 p.p.m. iron as iron tartrate added bi-daily throughout the growing period, the comparisons being made upon the basis of green and dry weights of tops and roots, organic nitrogen content, thickness of stems, extent of foliage, number of rootlets, and green coloring. The culture solutions containing iron humate do not accumulate the large precipitates of iron phosphate obtained with the customary forms of iron added, such as iron tartrate or sulfate. The concentration functions of the natural and synthetic iron humates with respect to both iron and humate fractions are substantially the same as those given by Azotobacter [figs. 2 and 3 and (11, fig. 2)]; synthetic humic acids containing 5 to 10 per cent Fe are required in smaller concentrations than the natural humic acids containing 1 per cent Fe; independently of the iron, the synthetic humate fraction becomes toxic at about 100 p.p.m. (as determined by using synthetic humic acids containing low contents of iron), whereas much higher concentrations of natural humic acids are required for toxicity by the humate fraction. More iron per unit of dry weight is required by Azotobacter than by tomato plants, but less dry weight is produced per unit volume of culture solution, so that the actual iron requirements per unit of culture solution are about the same. On the whole, the synthetic humic acids are probably somewhat preferable to the natural ones, in view of their better known composition, although where traces of unknown elements, and not merely iron, are required, the natural acids may be preferable. In the experiments being reported upon, no appreciable growth took place unless iron in some form were added. Thus, in one experiment lasting 3 weeks, the dead withered seedlings which resulted in the case of the solutions to which no iron was added

weighed 0.08 gm. (dry weight), compared to 1.22 and 2.51 gm. given by 0.02 and 2.0 p.p.m. iron as synthetic iron humate, or 2.24 gm. given by 11 p.p.m. iron as iron tartrate added in 11 bi-daily dosages of 2 p.p.m. iron each.

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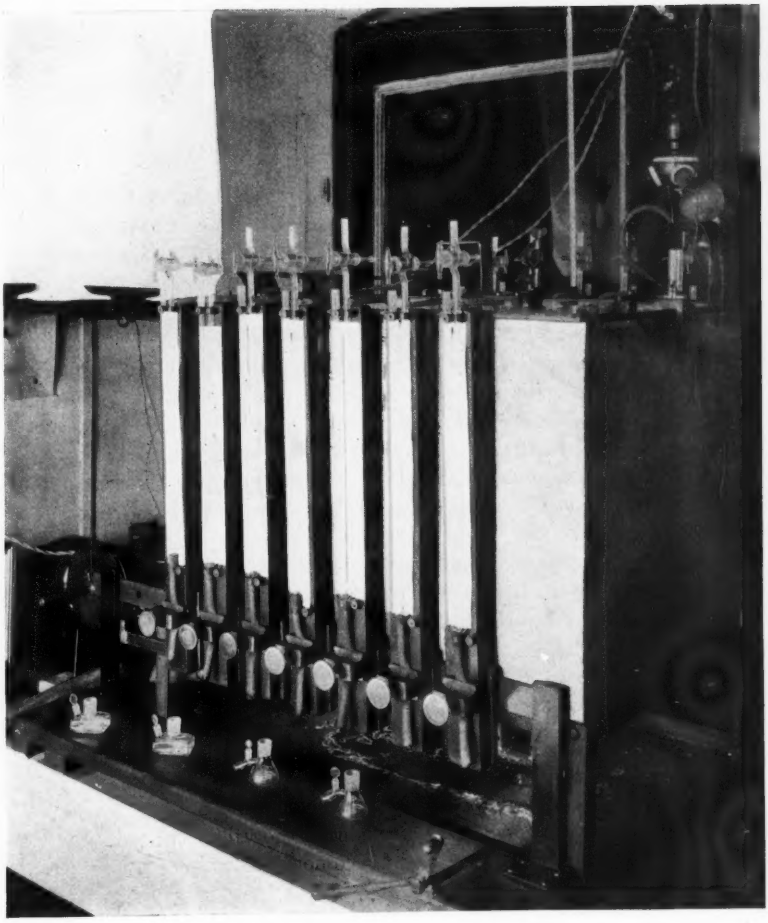
PLATE 1

WARBURG MICRO-RESPIRATION APPARATUS FOR MEASURING OXYGEN CONSUMPTION AND GROWTH

The cultures are contained in vessels (see figure 1) submerged in the constant temperature water bath. The vessels (not visible) are attached to the manometers, which are shaken at the rate of 120 cycles per minute with an amplitude of 3 cm. Fourteen manometers are accommodated in the apparatus, making a large number of simultaneous experiments possible. The temperature of the thermostat is generally maintained at 28° ±0.01°C. or 31° ±0.01°C.

PLATE 1



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THE PHYSIOLOGICAL NATURE OF HUMIC ACID STIMULATION OF AZOTOBACTER GROWTH

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This paper will present an analysis of various physiological aspects of stimulation of growth of Azotobacter caused by humic acid and substances capable of substituting for humic acid. Emphasis will be laid chiefly upon the relations obtaining immediately subsequent to application of the stimulant. Apart from our previous work reported casually in other connections (2, 3, 4, 5, 8), and the work of Iwasaki (11), little attention has been paid to these dynamic, differential relations. Most other investigations, including our own preceding one (8), have been concerned experimentally chiefly with effecting by means of humic acid large integral increases of growth in cultures grown for days or weeks. Functional relationships based upon velocities obtained in this manner are subject, however, to the contingent disadvantage that the duration of the experiment is a variable and often uncontrolled factor involving secondary incidental phenomena (2, p. 1177; 4, p. 390). In order to eliminate this objectionable feature, the velocity *constant* of growth will frequently be employed here, in preference to the velocity of growth. Correspondingly, the "humic acid velocity constant ratio" will be employed in preference to the "humic acid ratio," as a quantitative criterion of stimulation. Since growth in young cultures of Azotobacter maintained under constant conditions is logarithmic, functions based upon the former ratio will be independent of time factors, whereas functions based upon the latter ratio will vary with time (see table 1 and discussion thereof, "The Theoretical Integral Function").

EXPERIMENTAL METHOD

The preparation and description of the various humic acids employed, and the general experimental procedure, are described in the preceding paper (8). It may be recalled that the monomolecular velocity constant of growth is obtained by plotting natural logarithm of growth with respect to time, which yields a line the slope of which represents the constant g in the formula "$g = d\ ln\ (a + y)/d\ t$", where a is the initial amount of inoculum and y is the growth increase after t, the time in hours; g is an important fundamental criterion of growth since it represents *at all times* the rate of growth at unit concentration of cells, as distinguished from the observed velocity, which varies with the

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concentration and therefore with the time. (For further discussion of the velocity constant g, see 6, 14.) The g values reported in this paper will be based upon not one, but several values of y at respective t values, in order to average out minor experimental errors causing apparent departures of growth from being strictly logarithmic. The terms $g_{H.A.}$ and g_o will refer to the velocity constants of growth in the presence and absence of humic acid (or substitutes of humic acid) respectively, the ratio between the two being the humic acid velocity constant ratio (H. A.-V. C. R.).

THE HUMIC ACID CONCENTRATION FUNCTIONS

The differential functions

The concentration of natural humic acids yielding approximately optimum stimulation varies from 25 to 100 p.p.m., 90–100 per cent of the optimum being obtained most frequently at about 50 p.p.m., as shown in figure 1. This statement applies to experiments so conducted that significant decreases in the concentrations of the active fraction of humic acid do not occur during the period of measurement, i.e., the humic acid velocity constant ratio remains constant with respect to time, for any given concentration. Functions so obtained represent experiments in which the amount of growth measured is in effect infinitesimally small, since the time is also, in effect, infinitesimally small, and consequently they have been termed "differential." The integral functions represent the converse situation, where growths and durations are finite.

The corresponding concentration of iron contained in humic acid, yielding optimum stimulation, is about 0.2–0.8 p.p.m. The synthetic humic acids may be prepared to contain at least two to three times the amount of iron found in our preparations of iron-rich natural humic acids (0.75–1.5 per cent), and the "differential" optimum concentration is correspondingly lower, 5–25 p.p.m. (see fig. 3A).

The natural humic acid differential concentration function passes through a maximum at 200–600 p.p.m., as may be gathered from figure 2, curve V, and the corresponding synthetic humic acid function is correspondingly lower, about 100–300 p.p.m. We are of the opinion that the decreased stimulation occurring at higher concentrations is not due chiefly to iron, but for the most part to the organic fraction of the humic acid, inasmuch as synthetic humic acids containing practically no iron behave somewhat similarly.

Because of solubility limitations, it is ordinarily impossible to attain a concentration of inorganic iron sufficient to yield a differential optimum concentration, but the theoretical value is probably about 0.5 p.p.m. of *soluble* inorganic iron.

As may be gathered from fig. 3B, curve 4E (and to some extent from curves 5, 7B) the implied differential optimum concentration of ferric citrate is 2.5 p.p.m., or about 0.5 p.p.m. ferric citrate iron.

As seen in figure 1, the optimum H. A.-velocity constant ratios vary, in the case of young vigorous cultures, from 1.25 to 1.50. This has been our experience also in a large number of miscellaneous unreported experiments. The ratio may become somewhat higher at times, but only when the g value at 0

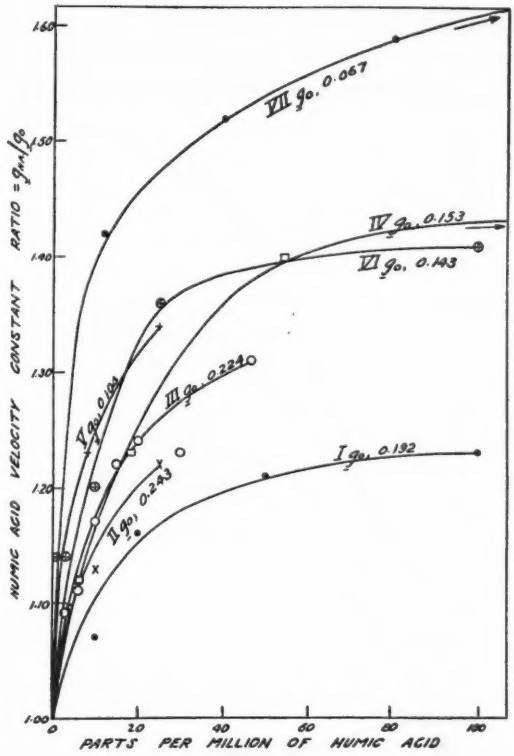

FIG. 1. THE DIFFERENTIAL INFLUENCE OF CONCENTRATION OF NATURAL HUMIC ACID UPON THE HUMIC ACID VELOCITY CONSTANT RATIO (WARBURG TECHNIQUE)

The g values in the presence and absence of humic acid, used to obtain the H. A.-Velocity Constant Ratio, are based in every case upon hourly y and t values observed over a period of 8–15 hours. g_0, used in denoting the curves, refers to the g value at 0 p.p.m. humic acid. All experiments performed at 28°C. Curves I, II, III, IV, V, VI, VII, cultures aged, in days, respectively 1, 1, $\frac{3}{4}$, 1 (diluted 2 times), 1, 1, 4 (diluted 6 times); initial respiration (cmm. O_2/cc./hour) respectively 4, 20, 20, 8, 25, 25, 65. H. A.-I employed in all cases, except H. A.-II in curve V. Source of nitrogen: N_2, except in curves II and III, where 50 p.p.m. NH_3-N.

p.p.m. H. A. is very low, about 0.08 or less, corresponding to a generation time of about 8 or more (see "Constant Temperature Conditions"). It will be noticed that the various curves in figure 1 are in general higher as the g value at 0 p.p.m. is lower, i.e., an inverse function obtains. This fact is highly

significant and is commented upon further in the section "The Influence of Velocity of Growth in the Absence of Humic Acid upon the Humic Acid Velocity Constant Ratio."

The integral functions

As the physiologically active fraction of the humic acid is consumed during the time course of an experiment, the observed "integral" optimum concentra-

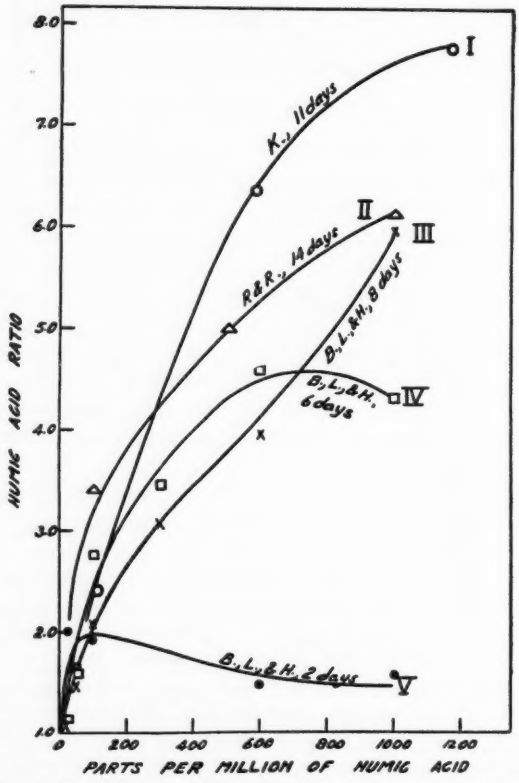

FIG. 2. THE INTEGRAL INFLUENCE OF CONCENTRATION OF NATURAL HUMIC ACID UPON THE HUMIC ACID RATIO IN EXPERIMENTS OF VERY LONG DURATION (ERLENMEYER TECHNIQUE)

Curve I. Data of Krzemieniewsky (13, p. 1004). Cultures grown in 200 cc. 1½ per cent glucose medium in large flasks. Growth measured as nitrogen fixed. Source of nitrogen: N_2. (The concentration of humic acid refers, in this single instance, to potassium humate.)

Curve II. Data of Remy and Rösing (15, p. 354). Cultures grown in 100 cc. 2 per cent mannite medium in large flasks. Growth measured as nitrogen fixed. Source of nitrogen: N_2.

Curve III, IV, and V. Data of Burk, Lineweaver, and Horner. Respectively 2, 6, and 8-day old cultures of the same experiment. H. A.-V employed. Each point represents an average of duplicates. 2 per cent glucose employed instead of 1 per cent (in anticipation of the length of the experiment). Final pH: 6.8 ± 0.1. Source of nitrogen: 200 p.p.m. NO_3-N.

tion will obviously shift higher and higher. When completely consumed, and the logarithmic phase of growth has been passed through, the growth velocity constant gradually becomes relatively less, per unit of cell concentration, compared to the control, as a result of consumption of the necessary elements of the medium, viz., Ca, P, Mg, glucose, not to mention Fe.

Figure 2 shows that in experiments of several days or weeks duration the integral optimum concentration may increase to 1,000–1,500 (and –2,000) p.p.m. natural humic acid. It is this type of concentration function which is usually reported in the literature; figure 2 represents exemplary data of other investigations [Krzemieniewsky (13), Remy and Rösing (15)], in addition to our own, which, it will be observed, are very similar. It is obvious that in representing integral functions for cultures several days old the humic acid ratio and not the velocity constant ratio is to be used.

Curves V, IV, and III of figure 2 represent a continuous experiment and show excellently the shifting of the integral optimum concentration with time. The optimum values are 50–300, 600–800, and greater than 1,000 (probably 1,500) p.p.m. at respectively 2, 6, and 8 days growth. Even the upward concavity developing in curve III is typical. It occurs because in relatively long-time experiments, because of limitation by other secondary factors, the humic acid ratio as a function of time passes through a maximum for sub-optimal amounts of humic acid [see (8, table 15)]. This effect is relatively greater the smaller the concentration. The general result is that the sub-optimal portion of the integral humic acid ratio concentration function changes with time from being first concave downward, to being relatively linear, to being finally concave upward, when the cultures are grown in unshaken flasks and no renewal of the medium is made. It will be noticed in this connection that, as might be expected, curve III lies, for the most part, below curve IV, even though the latter represents a younger culture. The actual growths in the case of curve III are, of course, greater; only the humic acid ratios are smaller, having been calculated, as usual, upon the basis of the growth in the 0 p.p.m.-culture for the respective day.

The integral concentration functions of synthetic humic acids and organic and inorganic iron behave qualitatively much the same as those already described in connection with natural humic acid. Comparing, for instance, in figure 3A, curves B, 11A, and those remaining, one notes that the synthetic humic acid optimum shifts from about 5 to 10, 30 to 50, to greater than 60 p.p.m., at 13 hours, 18 hours, and 2–11 days, respectively.

Mixed functions

In figures 3, 3A, and 3B are represented a number of miscellaneous experiments, including all the appertaining ones reported in the preceding paper (8). These will provide additional evidence with respect to the various relations discussed. In general, the experiments represented are of an intermediate type. They have been conducted for a period of time either somewhat too long or somewhat too short, respectively, to demonstrate either the minimum

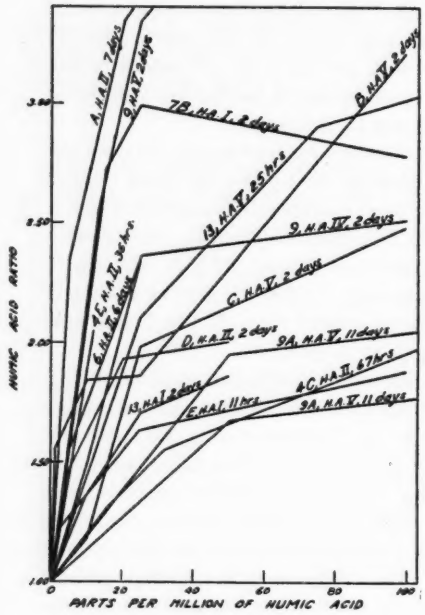
HUMIC ACID RATIO
PARTS PER MILLION OF HUMIC ACID
A, H.A.I. 7 days
9, H.A.V. 2 days
7B, H.A.I. 2 days
B, H.A.V. 2 days
13, H.A.V. 25 hrs.
9, H.A.IV. 2 days
4C, H.A.II. 36 hrs.
6, H.A.V. 6 days
C, H.A.V. 2 days
D, H.A.II. 2 days
9A, H.A.V. 11 days
13, H.A.I. 2 days
E, H.A.I. 11 hrs.
4C, H.A.II. 67 hrs.
9A, H.A.V. 11 days

FIG. 3

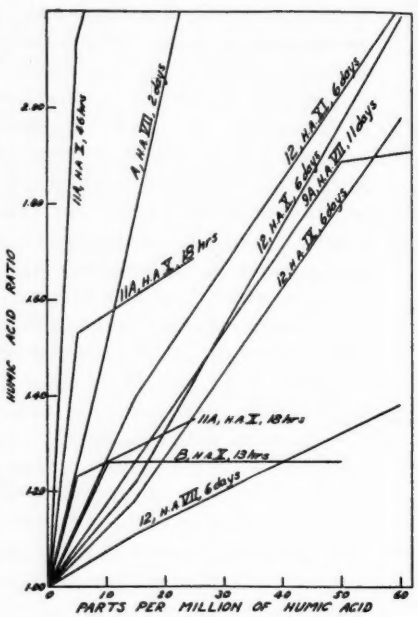
HUMIC ACID RATIO
PARTS PER MILLION OF HUMIC ACID
11A, H.A.X. 48 hrs.
A, H.A.VII. 2 days
12, H.A.XI. 6 days
12, H.A.X. 6 days
9A, H.A.VII. 11 days
12, H.A.IX. 6 days
11A, H.A.X. 18 hrs.
11A, H.A.X. 18 hrs.
B, H.A.X. 13 hrs.
12, H.A.VII. 6 days

FIG. 3A

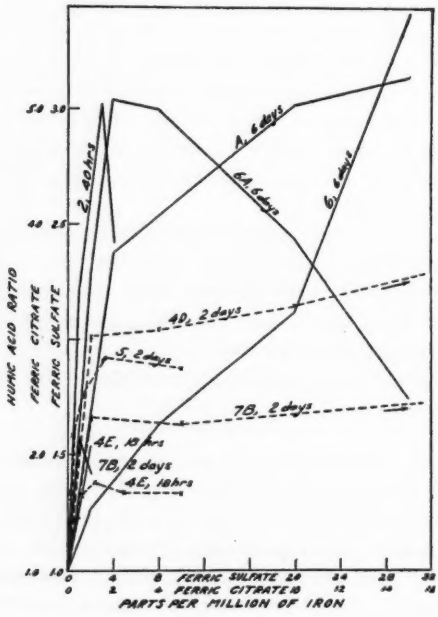

FIG. 3B

differential optimum concentration or the maximum integral optimum concentration.

Experiment 9A in figures 3 and 3A is possibly exceptional in being still concave downward, but since 2 per cent glucose was not employed in this very long experiment the energy supply must have imposed an important limiting secondary factor preventing the higher humic acid concentrations from exerting their usual effect. The last point on curve 7B (100 p.p.m.) is probably low. The difference of behavior of curves A, 6, and 6A, fig. 3B, all representing 6-day experiments with inorganic iron, illustrates the considerable latitude of variation which must often be expected in the exact time relations of relatively old cultures.

The theoretical integral function

It is evident from figures 2, 3, 3A, and 3B that the integral humic acid ratio rarely exceeds 8 to 10, even in long-time experiments, and that, in experiments of not more than a week's duration, values of 2 to 4 prevail as a rule. Were it not for such secondary factors as those discussed in the section "The Integral Functions", viz., consumptions of active fractions of humic acid, inorganic nutrients, energy supply, etc., much higher ratios might be expected, as shown in table 1. This is true in spite of the additional factor that a culture can obviously become only so heavy, through purely physical limitation.

In table 1 is represented the theoretical humic acid ratios which would obtain if by some means physiological consumption factors and physically limiting factors could be eliminated, and optimum humic acid velocity constant ratios could be maintained indefinitely. Guided by figure 1, we have given values for three different ratios, covering the normal range, 1.25, 1.50, and 1.75. Values are also given, in the case of each ratio, for three different velocity constants at 0 p.p.m. H. A., 0.175, 0.116, and 0.086. In order of magnitude the first two constants occur most frequently in *Warburg* technique

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FIG. 3. THE MIXED INTEGRAL-DIFFERENTIAL INFLUENCE OF CONCENTRATION OF NATURAL HUMIC ACID UPON THE HUMIC ACID RATIO

Curves A, B, C, D, represent turbidity measurements. Curve E represents respiration rate measurements and is taken from the same experiment reported in figure 1, curve VI. The other curves correspond to respective tabular data in the preceding paper (8).

FIG. 3A. THE MIXED INTEGRAL-DIFFERENTIAL INFLUENCE OF CONCENTRATION OF SYNTHETIC IRON-CONTAINING HUMIC ACID UPON THE HUMIC ACID RATIO

Curves A and B represent respiration rate measurements. The other curves correspond to respective tabular data in the preceding paper (8).

FIG. 3B. THE MIXED INTEGRAL-DIFFERENTIAL INFLUENCE OF CONCENTRATION OF INORGANIC AND ORGANIC ACID IRON UPON THE HUMIC ACID RATIO

Solid lines, ferric sulfate; broken lines, ferric citrate. Curve A represents turbidity measurements. The other curves correspond to respective tabular data in the preceding paper (8). To convert abscissal values to p.p.m. of iron "compound" multiply by 5.

experiments, the latter two in *Erlenmeyer* technique experiments, at 28° – 31°C. It is seen that even after only about 24 hours ratios greater than 8 to 10 may be expected, becoming 20 to 60 after 2 days, and attaining absurd values in 3 to 5 days or more.

Table 1 illustrates the relation already mentioned, that when the H. A.-velocity constant ratio is kept constant with respect to time the H. A.-ratio increases very rapidly with time, especially after about 12 hours, the increase

TABLE 1

The theoretical humic acid ratio as a function of time, at a maintained optimum constant humic acid velocity constant ratio

	H. A.-V. C. RATIO								
	1.25			1.50			1.75		
Generation time (G. T.) (in absence of H. A.).	8	6	4	8	6	4	8	6	4
Corresponding velocity constant (in absence of H. A.).	0.086	0.116	0.175	0.086	0.116	0.175	0.086	0.116	0.175
Theoretical humic acid ratio									
Time, *hours* 0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
4	1.09	1.12	1.19	1.19	1.26	1.42	1.29	1.41	1.69
8	1.19	1.26	1.42	1.41	1.58	2.02	1.67	2.00	2.85
12	1.29	1.41	1.69	1.67	2.00	2.87	2.15	2.82	4.80
24	1.67	2.00	2.86	2.78	3.98	8.13	4.63	7.94	23.2
48 (2 days)	2.78	3.98	8.13	7.73	15.8	66.8	21.5	63.1	537
120 (5 days)	12.9	31.6	191	167	200	355	2,090	3×10^4	6×10^6
168 (1 week)	35.8	126	1,560	1,280	2×10^6	2×10^6	5×10^4	2×10^6	3×10^9
336 (2 weeks)	1,280	2×10^4	2×10^6	2×10^6	3×10^8	6×10^{12}	2×10^9	4×10^{12}	1×10^{19}

being logarithmic. It is also true that for a given period of time and for a given H. A.-velocity constant ratio the H. A.-ratio likewise increases logarithmically with the magnitude of g_o.

THE INFLUENCE OF VELOCITY OF GROWTH IN THE ABSENCE OF HUMIC ACID UPON THE HUMIC ACID VELOCITY CONSTANT RATIO

Constant temperature conditions

It has been shown (8) that humic acid is a stimulant but not an essential for growth, and that it adds iron in a form more soluble and highly available

than the usual inorganic iron supplied. It would seem reasonable to expect, therefore, that as the growth velocity is increased, from one cause or another, humic acid stimulation will decrease, since there is less opportunity for stimulation; a maximum possible velocity is being approached which is determined by numerous other factors (glucose concentration, salt concentration, temperature, etc.) which are, on the whole, unrelated to the stimulating agent. At low velocities the ratio (maximum possible velocity)/(low velocity) is much higher than at high velocities and opportunity for stimulation presumably much greater.

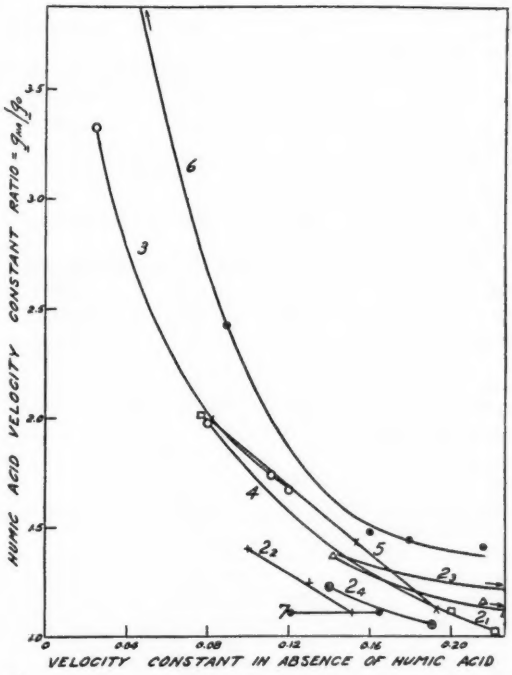

FIG. 4. STIMULATION AS A FUNCTION OF GROWTH VELOCITY
The curves are lettered corresponding to the data given in tables 2–7 inclusive

The inverse relationship between growth velocity and stimulation is obtained, in fact, with respect to all factors regulating growth velocity under conditions of constant temperature, as follow: nitrogen source; pressure of nitrogen gas; concentration of fixed nitrogen; medium constitution; and age or heaviness of culture; as shown, respectively, in tables 2, 4, and 5; 3; 4; 5; and 6. Figure 4 presents a plot of all the functions.

The close similarity in form and position of the curves in figure 4 is somewhat

surprising. They all fall between the limits of a narrow two-dimensional hyperbola which tends to approach the abscissa and ordinate practically within experimental error of measurement at, respectively, g_o = approximately 0.2, and H. A.-velocity constant ratio = approximately 5. It would appear that the latter ratio rarely exceeds 1.15 − 1.20 when the velocity constant in

TABLE 2

The influence of growth velocity (as governed by nitrogen source) upon humic acid stimulation (Warburg technique)

Experiment 1:				
Relative velocity constant (in absence of H. A.)	1.00	1.35	1.41	1.55
Source of nitrogen (fixed nitrogen)	N_2	NO_3-N (50)	NH_3-N (50)	Urea-N (50)
g, no humic acid	0.160	1.216	0.226	0.248
g, 50 p.p.m. H. A.-V	0.202	0.248	0.250	0.258
H. A.-velocity constant ratio	1.26	1.15	1.10	1.04
Experiment 2:				
Relative velocity constant (in-absence of H. A.)	1.00	1.30	1.50	.....
Source of nitrogen (fixed nitrogen: p.p.m.)	N_2	NO_3-N (50)	NH_3-N (100)	.....
g, no humic acid	0.100	0.130	0.150	.....
g, 25 p.p.m. H. A.-II	0.140	0.160	0.165	.....
H. A.-velocity constant ratio	1.40	1.24	1.10	.....
Experiment 3:				
Relative velocity constant (in absence of H. A.)	1.00	1.70	.....	.....
Source of nitrogen (fixed nitrogen: p.p.m.)	N_2	NH_3-N (50)	.....	.....
g, no humic acid	0.143	0.243	.....	.....
g, 25 p.p.m. H. A.-II	0.195	0.296	.....	.....
H. A.-velocity constant ratio	1.36	1.22	.....	.....
Experiment 4:				
Relative velocity constant (in absence of H. A.)	1.00	1.38	.....	.....
Source of nitrogen (fixed nitrogen: p.p.m.)	N_2	NO_3-N (50)	.....	.....
g, no humic acid	0.140	0.193	.....	.....
g, 40 p.p.m. H. A.-I	0.172	0.203	.....	.....
H. A.-velocity constant ratio	1.23	1.05	.....	.....

Experiments 1, 2, 3, 4, respectively: 1, 1, 1, 2 days old; 31°, 27°, 28°, 28°C.; final pH 6.7, 7.0, 6.8, 7.0 ± 0.1 (except in NH_3-N cultures 6.6 ± 0.2).

the absence of humic acid exceeds 0.14, from whatever cause. Conversely, the ratio generally exceeds 2.00 when the constant falls below 0.08 to 0.10, and increases still further very rapidly as the constant becomes still smaller.

In some, but not all, of the appertaining tables, somewhat larger stimulation is obtained in free than in fixed nitrogen, but this is what would be expected,

quantitatively, in view of the correspondingly somewhat smaller growth velocity and also the differences between other forms of fixed nitrogen. There is no need to consider that humic acid is exerting any significant additional

TABLE 3

The influence of growth velocity (as governed by nitrogen gas pressure) upon humic acid stimulation (Warburg technique)

Relative velocity constant (in absence of H. A.)	1.00	1.24	4.48	4.80
N_2 pressure (atm.)	0.10	0.25	0.45	0.78
g, no humic acid	0.025	0.081	0.112	0.120
g, 125 p.p.m. H. A.-II	0.083	0.160	0.195	0.200
H. A.-velocity constant ratio	3.32	1.97	1.74	1.66

Thirty-six-hour-old *A. chroococcum*. 0.20 atm. O_2 in different pressures of H_2 and N_2 to 1 atm. total pressure.

TABLE 4

The influence of growth velocity (as governed by supra-optimal concentration of fixed nitrogen) upon humic acid stimulation (Warburg technique)*

Relative velocity constant (in absence of H. A.)	2.91	2.63	1.00	(1.91)
Concentration of NO_3-N (p.p.m.)	625	1,250	5,000	(N_2-gas)
g, no humic acid	0.221	0.200	0.076	(0.145)
g, 50 p.p.m. H. A.-II	0.225	0.221	1.54	(0.199)
H. A.-velocity constant ratio	1.02	1.12	2.02	(1.37)

Eighteen-hour-old *A. vinelandii*. Final pH 7.0 ± 0.2.

* Supra-optimal rather than infra-optimal concentrations were employed—on account of the much greater ease in keeping the given concentrations constant throughout the course of the experiment.

TABLE 5

The influence of growth velocity (as governed by nature of medium) upon humic acid stimulation (Warburg technique)

Relative velocity constant (in absence of H. A.)	2.42	1.85	1.00
Source of nitrogen (fixed nitrogen: 50 p.p.m.)	NO_3-N	NO_3-N	N_2
Medium*	Customary	Phosphate	Customary
g, no humic acid	0.192	0.153	0.083
g, 50 p.p.m. H. A.-II	0.216	0.220	0.170
H. A.-velocity constant ratio	1.13	1.44	2.00

* Thirty-six-hour-old *A. vinelandii* diluted 6-fold with either customary (i.e., complete nutrient) medium or 0.1 per cent potassium phosphate (i.e., without Ca, Mg, Na, Fe, Cl, SO_4). 1 per cent glucose added to both media. Final pH 6.9 ± 0.1.

influence upon the mechanism of nitrogen fixation, although it may at times do so (6); the experiments of table 6 are possibly also an exception to this statement. As shown in table 4, when the growth velocity in nitrate-nitrogen

is lowered by excessive nitrate concentration to a value lower than the growth velocity in free nitrogen, the stimulation is then correspondingly greater. Moreover, the stimulation depends markedly upon the nitrogen pressure (table 3), and in table 5 stimulation in nitrate nitrogen is affected considerably by lowering the concentration of the common inorganic constituents, apart from potassium phosphate.

TABLE 6

The influence of growth velocity (as governed by heaviness of culture) upon humic acid stimulation (Warburg technique)

Relative velocity constant (in absence of H. A.)	7.2	6.0	5.3	2.9	1.00
Relative degree of heaviness of culture	1.0	2.5	3.1	4.4	7.5
Heaviness of culture (initial respiration rate) (cmm. O_2/cc./hour)	16	40	50	70	120
g,* no humic acid	0.216	0.180	0.160	0.089	0.030
g, 50 p.p.m. H. A.-V	0.302	0.258	0.236	0.215	0.136
H. A.-velocity constant ratio	1.40	1.43	1.48	2.42	4.53

Five 1-day-old *A. vinelandii* cultures which had been inoculated 24 hours previously with different amounts of inoculum so that after 24 hours different amounts of growth would be obtained. 31°C. Final pH 6.7 ± 0.1 Source of nitrogen: N_2.

* With the possible exception of the lightest, or two lightest, cultures, the growth velocities in the absence of humic acid were no longer strictly logarithmic, but tended to fall off gradually, chiefly because of incipient iron deficiency; consequently the average velocity for about second-eighth hour is employed. This procedure introduces only a slight uncertainty; the general conclusion is sufficiently established by this experiment, and many others not reported. Upon addition of humic acid, all cultures gave logarithmic growths. Whereas the differences in slope between the no-humic acid cultures are due chiefly to iron (and secondarily to other factors), the differences in the humic acid cultures are due chiefly to other factors (such as calcium, possibly phosphate, sugar, etc.)

TABLE 7

*The influence of growth velocity (as governed by glucose concentration) upon humic acid stimulation** *(Warburg technique)*

Relative velocity constant (in absence of H. A.)	1.00	1.35
Initial glucose concentration (per cent)	0.1	1.1
g, no humic acid	0.122	0.165
g, 50 p.p.m. H. A.-V	0.132	0.178
H. A.-velocity constant ratio	1.11	1.11

* Data taken from table 11, fifth to thirteenth hour. Source of nitrogen: N_2.

It is seen in table 7 that although the growth velocity is 1.35 times as great in 1.1 per cent as compared to 0.1 per cent glucose, the H. A.-velocity constant ratio has not varied appreciably. Although it should not have been expected to vary markedly, in view of the magnitude of *g* and ratio values involved, approaching 0.14 and 1.0 − 1.1, respectively, an even more important circumstance lies in the fact that at the low glucose concentration energy supply (oxy-

gen consumption) is rapidly becoming the chief limiting factor of growth and humic acid stimulation could not be expected in great degree.

Apart from tables 2–7, inclusive, and figure 4, further evidence in favor of the inverse relation between growth velocity and H. A.-velocity constant ratio may be obtained from figure 1 and figure 5, and from considerations of the effect of age of culture. In figure 1 the curves group themselves roughly in order of growth velocity in the absence of humic acid. In figure 5 stimulation is greater in nitrogen gas than in urea-nitrogen at all temperatures; at the same time the growth velocity is smaller at all temperatures (6, 14). We have observed that, in general, as the age of a culture increases the velocity constant decreases and the stimulation increases in much the same manner as shown in table 6, where

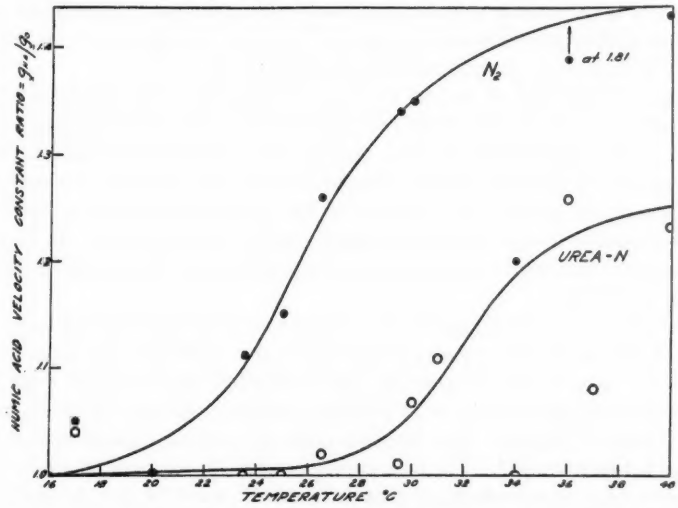

FIG. 5. THE INFLUENCE OF TEMPERATURE UPON THE HUMIC ACID-VELOCITY CONSTANT RATIO (WARBURG TECHNIQUE)

differences in heaviness of culture are obtained by using different-sized inocula, rather than varying the age.

Iwasaki (11) has shown that as the oxygen pressure is lowered from 21 per cent to about 2–4 per cent, and the growth rate increases, the effect of humic acid decreases. This we have confirmed. Below 2–4 per cent oxygen the growth rate in the absence of humic acid again decreases, but humic acid has no effect here, nor in general does fixed nitrogen or other factors, since energy supply (oxygen consumption) is now more or less the completely limiting factor of growth rate.

The inverse relationship obtaining at any given constant temperature between growth velocity and stimulation may be interpreted as rather strong

additional evidence that natural humic acid is to be classed as a stimulator of growth. Iron in *some* form is essential for growth, but the ability of natural humic acid to supply this iron is not specific. Another manner of regarding the matter is to consider that if iron is one of the chief factors controlling growth velocity, especially at the lower velocity, then the fact that one culture is growing more rapidly than another (with the same original iron supply) indicates empirically that less iron is needed in the former case and hence less stimulation could be produced.

The influence of temperature

The temperature function behaves to some extent conversely to the constant temperature growth velocity function just reported. Humic acid stimulation *increases* as the growth velocity is increased as a function of temperature over the *sub-optimal* growth velocity range (10° — 33°C. in N_2, and 10° — 33° — 37°C. in various forms of fixed nitrogen, see 6, 7, 14).

Figure 5 and table 8 present the data in connection with the influence of temperature upon the humic acid velocity constant ratio over the range 16° — 40°C. The experiments reported therein were performed in quite another connection (6, 14) and although the smoothed curves of figure 5 represent a few discrepant points, the existence of the temperature function, in rough approximation to that indicated, would appear unquestionable. It may be mentioned that the growth temperature limits of Azotobacter are approximately 10°C. and 50°C.

It will be observed in figure 5 that below a certain temperature humic acid stimulation of growth velocity is negligible (about 20°C. in N_2, about 26° in urea-N). Above this temperature the stimulation ratio becomes finite and increases through some 10 to 12 degrees to attain a relatively constant value for possibly 5 degrees. The function takes the general form of an S-shaped curve between 0–40°C. The urea curve, as would be expected in view of the considerably greater velocity of growth in urea-N (see 6, 14, and the preceding section "Constant Temperature Conditions"), is considerably lower than in N_2. No respective data are available, but curves for NH_3-N, NO_3-N, and NO_2-N would undoubtedly lie intermediate, in view of their intermediate growth velocities (6, 14).

Table 8 brings out other interesting relations. The induction time is substantially independent of temperature, respiration rate at which stimulation commences, and the H. A.-velocity constant ratio. Likewise, the last-named function, although dependent upon temperature, is substantially independent of the respiration rate at which stimulation commences (12–90 cmm. O_2/cc./hr.) and, as just stated, the induction time, at least over the infra-optimal temperature range. The 2-hour induction values in N_2, above 33°C., may be significantly lower than values at lower temperatures.

The reason for sub-optimal temperatures yielding a humic acid velocity constant ratio function of g_o the converse of that obtained in the case of all

other investigated factors is not entirely clear. It is possible that temperature, as contrasted to all other factors, possesses some direct influence upon the permeability of humic acid, or upon its maintained concentration within the cell. It is very probable (6) that iron and temperature are the chief external factors regulating growth velocity under usual conditions and that increasing temperature increases not only growth velocity but also the relative need for iron

TABLE 8

The influence of temperature upon the humic acid-velocity constant ratio in free and urea nitrogen (Warburg technique)

	SOURCE OF NITROGEN					
	N_2			Urea		
Factor.....	Induction* time	Respiration rate at end† of induction (cmm. O_2/cc./hour)	H. A.-velocity constant ratio	Induction* time	Respiration rate at end† of induction (cmm. O_2/cc./hour)	H. A.-velocity constant ratio
Temperature °C.						
16	—	(9)	1.00	—	(10)	1.00
17	4	13	1.05	4	14	1.04
20	—	(18)	1.00	—	(19)	1.00
23.5	5	42	1.11	—	(35)	1.00
25	3	24	1.15	—	(24)	1.00
26.5	4	20	1.26	—	(22)	1.02
29.5	2	16	1.34	—	(28)	1.01
30	4	41	1.35	4	44	1.07
31	..	....	....	5	26	1.11
34	5	12	1.20	—	(14)	1.00
36	2	24	1.81	5	90	1.26
37	..	....	....	4	42	1.08
40	2	28	1.43	4	42	1.23

See (6, 14) for detailed description of the experiments. In brief, 1-day old cultures were invariably employed and growth measurements made for 7–8 hours.

* The time required for humic acid stimulation to become evident, i.e., for the humic acid ratio to become 1.03–1.05, and to continue to increase thereafter, or (again, i.e.) for the velocity constant in humic acid to definitely assume a new (and constant) value as represented by a new slope when "log. respiration rate" is plotted against time.

† i.e., the respiration rate when stimulation first commences. The values in parentheses in this column represent the respiration rates at an arbitrarily assumed time (fourth hour) in those cases where no stimulation occurs.

to maintain the increased velocity. In the case of experiments made concerning the other factors, iron and temperature were substantially optimum and were not limiting. There was no reason to suppose that variation in other factors could greatly increase or alter the iron need, even when the growth velocities were increased, since as pointed out in the first and last paragraphs of the section "Constant Temperature Conditions," such increases in growth

TABLE 9

The length of induction period given by natural humic acids in various experiments (Warburg technique)

INDUCTION* LENGTH	NITROGEN SOURCE (FIXED NITROGEN)	H. A.	g_0	$\varepsilon_{H.A.}$	$\frac{\varepsilon_{H.A.}}{g_0}$	RESPIRATION RATE AT BEGINNING OF STIMULATION	CULTURE† AGE	TEMPERATURE
hours	*p.p.m.*	*p.p.m.*				*cmm. O_2/cc./hour*	*days*	*°C.*
1	N_2	I-54	0.15	0.21	1.41	10	1	28
1	N_2	I-125	0.13	0.22	1.69	14	1	28
1	N_2	I-50	0.11	0.18	1.64	25	1(3)	28
2	N_2	II-50	0.07	0.12	1.67	86	2(4)	28
2	N_2	II-25	0.10	0.14	1.40	28	1	28
2	N_2	I-50	0.13	0.20	1.54	38	1	28
3	N_2	I-50	0.15	0.20	1.33	43	1	28
3	N_2	V-50	0.17	0.28	1.65	38	1 (2)	31
3	N_2	V-50	0.09	0.21	2.34	90	1	31
3	N_2	V-50	0.18	0.24	1.33	65	1	31
3	N_2	V-100	0.22	0.26	1.18	56	1	31
3	N_2	II-40	0.16	0.18	1.13	25	1	28
3	N_2	II-40	0.16	0.18	1.13	35	1	28
3	N_2	II-40	0.16	0.19	1.19	48	1	28
3	N_2	II-40	0.16	0.20	1.25	63	1	28
3	N_2	II-40	0.16	0.20	1.25	72	1	28
3	N_2	II-40	0.16	0.22	1.37	73	1	28
4	N_2	V-50	0.28	0.36	1.29	24	1	31
4	N_2	V-50	0.20	0.26	1.30	70	1	31
4	N_2	V-50	0.03	0.14	4.67	140	1	31
4	N_2	V-50	0.01	0.03	3.33	75	1	31
4	N_2	I-125	0.20	0.25	1.25	12	1	28
4	N_2	IV-25	0.12	0.19	1.58	43	1	28
4	N_2	IV-100	0.12	0.20	1.67	43	1	28
4	N_2	V-25	0.12	0.15	1.25	43	1	28
4	N_2	V-100	0.12	0.18	1.50	43	1	28
5	N_2	V-50	0.12	0.14	1.17	40	1	28
5	N_2	V-50	0.15	0.18	1.20	11	1	31
5	N_2	V-25	0.02	0.06	3.00	80	1	31
6	N_2	V-50	0.09	0.15	1.67	81	1	31
6	N_2	V-50	0.09	0.14	1.56	98	2	28
6	N_2	II-20	0.19	0.21	1.11	18	1	28
6	N_2	I-80	0.04	0.07	1.75	15	4 (6)	28
6	N_2	I-40	0.13	0.18	1.38	50	2 (3)	28
6	N_2	II-40	0.13	0.18	1.38	50	2 (3)	28
7	N_2	I-40	0.12	0.27	2.25	14	8 (27)	28
7	NH_3-N:50	II-50	0.22	0.28	1.27	96	1	28
9	NO_3-N:50	II-40	0.14	0.16	1.14	50	2 (4)	28
3‡	N_2	V-50	0.15	0.20	1.33	54	(1)	31
	NO_3-N:50		0.22	0.25	1.14	65		
	NH_3-N:50		0.23	0.26	1.13	70		
	Urea-N:50		0.25	0.26	1.04	75		

* See footnote, table 8.

† Subscript in parenthesis refers to initial degree of dilution, if any, with customary medium containing 1 per cent glucose.

‡ These bracketed experiments in free and fixed nitrogen performed simultaneously.

TABLE 9—*Concluded*

INDUCTION* LENGTH	NITROGEN SOURCE (FIXED NITROGEN)	H. A.	g_0	$g_{H.A.}$	$\frac{g_{H.A.}}{g_0}$	RESPIRATION RATE AT BEGINNING OF STIMULATION	CULTURE† AGE	TEMPERATURE
hours	*p.p.m.*	*p.p.m.*				*cmm. O_2/cc./hour*	*days*	*°C.*
3‡	N_2	II-50	0.10	0.17	1.70	12	1 (6)	28
	NO_3-N:50		0.10	0.22	1.38	13		
3‡	N_2	II-50	0.10	0.19	1.19	38	1	28
	NO_3-N:50		0.18	0.22	1.22	41		
8‡	N_2	I-125	0.19	0.23	1.21	20	1	28
	NH_3-N:50		0.26	0.37	1.42	25		
8‡	N_2	II-10	0.14	0.17	1.21	26	1	28
9	NH_3-N:50		0.23	0.30	1.30	66		

velocity tended toward a limiting maximum velocity determined chiefly by the other factors besides iron. In other words, the problem reduces to one of when iron is and is not the chief factor determining growth velocity and H. A.-velocity constant ratio.

THE INDUCTION PERIOD

Length

A certain length of time elapses after addition of humic acid to a culture before stimulation commences. This induction period varies, in general, from 1 to 10 hours, as shown in table 9. It is seen that 3 to 4 hours is the most frequent period, the distribution frequency being as follows: 1, 2, 3, 4, 5, 6, 7, 8, 9 hours, respectively, 3, 3, 19, 9, 3, 6, 2, 3, 2. Commencement of stimulation is defined as the time when the humic acid ratios commence to rise appreciably above 1, i.e., 1.03 to 1.05, and subsequent time values indicate, by increasing in value, that the values 1.03 to 1.05 were real, and did not represent experimental error. There has been occasional indication that induction values considerably greater than 9 hours occur sometimes in young, very highly diluted, rapidly growing cultures (see 8, tables 19 and 20). This is in some measure to be expected in view of the results shown in figure 4, which indicate that when a certain high velocity is reached, say g_o = about 0.2 − 0.25 (in the case of Azotobacter), substantially no humic acid stimulation is possible. The very prolonged onset of stimulation in such cases is therefore probably not induction, but simply *the absence of stimulation.*

Character of onset of stimulation

The onset of stimulation occurs rather sharply over a period of generally not more than one hour, as may be seen particularly in figure 6, but also in figures 7 and 8, and to some extent in tables 10 and 11. During induction $g_o = g_{H.A.}$, but at the onset of stimulation the new $g_{H.A.}$ is established

suddenly; this is true, as seen in figure 6, independently of the length of induction, i.e., 1, 2, 3, 4, 4, 5, 5, 5, or 8 hours, in curves III, V, IX, VIII, VI, IV, VII, II, and I, respectively.

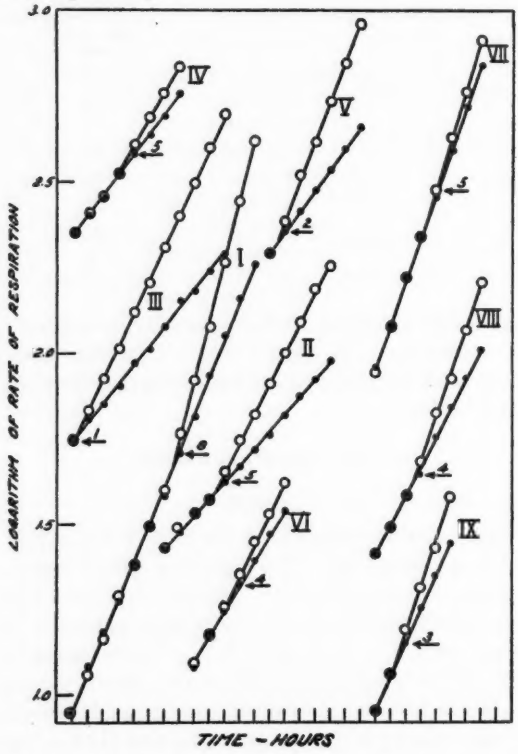

FIG. 6. THE SHARPLY DEFINED ONSET OF HUMIC ACID STIMULATION WITH DIFFERENT LENGTHS OF INDUCTION PERIOD, HUMIC ACIDS, TEMPERATURES, AND NITROGEN SOURCES (WARBURG TECHNIQUE)

Arrows indicate the approximate hour of definite commencement of stimulation and end of induction. In order to place all the nine stimulation curves in the figure the respiration rates have been calculated varyingly, per 1 cc., per 2 cc., etc.; this results in merely an ordinate shift of the entire curve. The cultures in the presence and absence of humic acid are indicated by circles and dots respectively. All cultures 1-day-old *A. vinelandii*, except No. IV, 2-day old.

Curve I: H. A.-I: 125 p.p.m.; 28°C.; per 2 cc; NH_3-N: 50 p.p.m.
Curve II: H. A.-II: 100 p.p.m.; 28°C.; per 1 cc.; NH_3-N: 50 p.p.m.
Curve III: H. A.-I: 125 p.p.m.; 28°C; per 4 cc.; N_2
Curve IV: H. A.-II: 40 p.p.m.; 28°C.; per 1 cc.; N_2
Curve V: H. A.-V: 60 p.p.m.; 36°C.; per 1 cc.; N_2
Curve VI: H. A.-V: 50 p.p.m.; 26.5°C.; per 1 cc.; N_2
Curve VII: H. A.-V: 50 p.p.m.; 31°C.; per 8 cc.; Urea-N: 100 p.p.m.
Curve VIII: H. A.-V: 50 p.p.m.; 31°C.; per 1 cc.; N_2
Curve IX: H. A.-V: 100 p.p.m.; 31°C.; per ½ cc.; N_2

TABLE 10

Comparison of induction periods of synthetic and natural humic acids (Warburg technique)

	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	H. A.-ratio					
Humic acid, 50 p.p.m.....	V (natural)	VII (synthetic)	V	VII	V	VII
hours						
Time, 1	1.00	1.00	1.00	1.00	1.00	1.00
2	1.00	1.00	1.01	1.02	1.00	1.00
3	1.00	1.00	1.14	1.02	1.00	1.00
4	1.02	0.99	1.23	1.11	1.01	1.00
5	0.99	1.02	1.42	1.15	1.01	1.00
6	1.01	1.01	1.52	1.22	1.02	1.04
7	1.06	1.04	1.57	1.26	0.99	1.06
8	1.07	1.05	1.89	1.41	1.02	1.07
9	1.13	1.11	2.59	1.99	1.16	1.13
10	....	....	2.95	2.15	1.17	1.22
12	1.17	1.18	....	....	....	....
13	1.17	1.20	....	....	....	....
14	1.20	1.24	....	....	....	....
16	....	....	3.56	3.06	1.28	1.36

In all experiments, 1-day-old cultures of *A. vinelandii*, pH 7.0 ± 0.1. Source of nitrogen: N_2. Initial respiration (cmm. O_2/cc./hr.). Experiments 1, 2, 3: 5, 15, 35.

TABLE 11

The influence of glucose concentration upon the length of induction period and humic acid ratio (Warburg technique)

	PER CENT GLUCOSE					
	0.12–0.15		1.12–1.15		H. A.-ratio	
50 p.p.m. H. A.-V.....		X		X	Low sugar	High sugar
Rate of respiration* (cmm. O_2/2 cc./hour)						
hours						
1	(12.4)	(8.6)	(11.6)	(8.0)	(−)	(−)
2	20.7	(20.7)	23.3	(23.3)	(1.00)	(1.00)
3	29.8	29.8	28.5	28.9	1.00	1.01
4	37.6	38.0	37.2	37.6	1.01	1.01
5	39.5	41.4	39.8	41.0	1.05	1.03
6	44.0	46.7	49.1	52.6	1.06	1.07
7¼	47.9	50.0	55.6	61.6	1.04	1.11
8¼	57.9	62.1	67.3	73.4	1.07	1.09
9¼	64.5	70.2	86.7	99.0	1.09	1.10
12	86.0	96.3	120	142	1.12	1.18
13	102	116	155	184	1.14	1.19

Thirty-six-hour-old *A. vinelandii* diluted 5 times with customary medium. pH 6.8 ±0.1.

* Averages of duplicate determinations.

Figure 7 shows that the sharpness of onset of induction, as well as the length of induction, is substantially independent of concentration of humic acid, although perhaps not entirely. In this figure, as well as in figure 8, the slope of any curve at any moment is proportional to stimulation as measured by "$g_{H.\,A.} - g_o$," or the increase in velocity constant caused by humic acid, as is evident from the following formula $g_{stim} = g_{H.\,A.} - g_o = ln\,((a + y_{H.\,A})/(a + y_o))/t = ln$ (H. A.-ratio)$/t$. If necessary, the humic acid velocity constant ratio may be obtained from the relation H. A.-V. C. R. $= (g_{stim} + g_o)/g_o$, g_o being given.

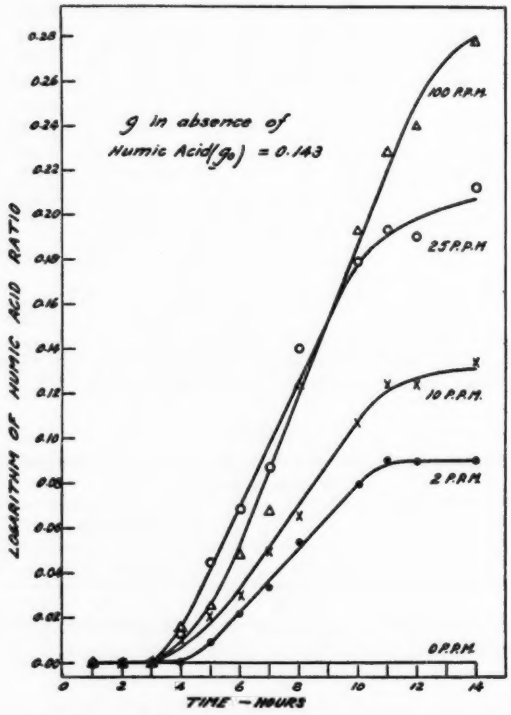

FIG. 7. HUMIC ACID STIMULATION AS A FUNCTION OF TIME AND CONCENTRATION OF HUMIC ACID (WARBURG TECHNIQUE)

One-day old *A. vinelandii*. 28°C. Final pH 7.0 ±0.1. H. A.-II employed. Source of nitrogen: N_2.

At all concentrations the induction periods in figure 7 last about 3 hours, and the new respective $g_{H.\,A.}$ values are established within 1 to 2 hours. It is possible that establishment in the case of 2 and 10 p.p.m. H. A. is somewhat longer than at 25 p.p.m. Triplication of cultures would be necessary to es-

tablish this minor point with certainty. It is definitely longer in the case of 100 p.p.m., even though the slope is slightly greater than at 25 p.p.m., because of the phenomenon, elsewhere referred to, that, at about 100 p.p.m. humic acid or greater, stimulation is at first inhibited slightly for secondary reasons, so that a combination of stimulation and temporary slight inhibition obtains.

The humic acid velocity constant ratio as a function of concentration, considered in figure 1, is well illustrated in figure 7 for the case of a single experiment. There is a gradual increase in $g_{H.A.}$ (i.e., $g_{H.A.} - g_o$) up to

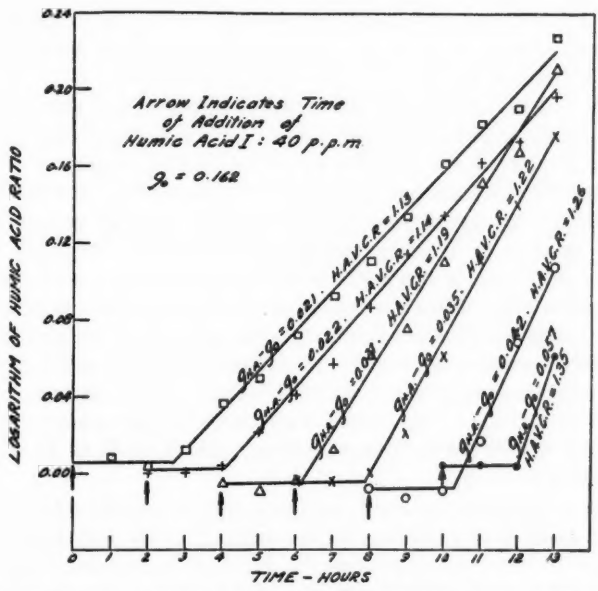

FIG. 8. THE INDUCTION PERIOD AS A FUNCTION OF MOMENT OF ADDITION (WARBURG TECHNIQUE)

One-day old *A. vinelandii*. 28°C. Final pH 6.9 ±0.1. 40 p.p.m. H. A.-II employed. Source of nitrogen: N_2. In this experiment a number of identical culture vessels were set up at $t = 0$, and humic acid added to each of two duplicate cultures at $t = 0$, 2, 4, 6, 8, and 10 hours, respectively.

about 25–100 p.p.m. humic acid. The consumption of the active stimulating fraction of humic acid is also well illustrated in figure 7 by the ultimate decreases in slope to zero at 10–14 hours, occurring perhaps a little more sharply and sooner, the lower the concentration. The assumption of a horizontal or zero slope indicates that the active fraction of the humic acid has been entirely consumed and that the culture is then growing with the same velocity constant as the culture in the absence of humic acid.

The cause of induction

We have been unable to ascertain the chief factor determining length of induction period, but, as may be gathered particularly from table 9, and to some extent from tables 8, 10, 11, and figures 6, 7, 8, a great many factors have been investigated with negative results in every case. The length of induction period would appear to be substantially independent of temperature (table 8); source of nitrogen (tables 8, 9; fig. 6); sugar concentration (Table 11); humic acid preparation (Tables 9, 10; fig. 6); concentration of humic acid (table 9; figs. 6, 7); age of culture or degree of dilution of culture (table 9); respiration rate at onset of stimulation (tables 8, 9, 10; fig. 6); moment of addition (fig. 8); g_o (table 9; fig. 6); $g_{H.\,A.}$ (table 9; fig. 6); and H. A.-velocity constant ratio (table 9, fig. 6).

Plots of data in table 9 of induction period length against either g_o, $g_{H.\,A.}$, $g_{H.\,A.}/g_o$, or "respiration rate at beginning of stimulation" yield no detectable function; the points are scattered in an entirely random manner.

The experiment represented in figure 8 was performed in order to eliminate the possibility that the observed induction period occurred because of some property peculiar to *Warburg* technique experiments. According to this possibility cultures so grown might require a certain period of adjustment in the apparatus before being susceptible to stimulation. It is seen, however, that this possibility does not obtain. As the moment of addition of humic acid to the culture is varied from 0, 2, 4, 6, 8 and 10 hours the induction period remains approximately constant at 3 to 4 hours, and then, in every case, the new velocity constant of stimulation is established within an hour or two. It is of interest to note the slight increase of $g_{H.\,A.}$ and H. A.-V. C. R. with time or moment of addition.

In view of the aforementioned negative evidence with respect to so many factors it would seem probable that the existence of the induction period is determined by some purely physiological factor such as permeability or rate of entrance of the active stimulating fraction of humic acid into the cells, which is beyond the scope of this paper to investigate.[2]

[2] Iwasaki (11), working in Meyerhof's laboratory, has also observed that at 28°C. a sharply defined induction period occurs which is relatively independent of the concentration of humic acid; that the action of humic acid is due to the consumption of some constituent, since the length of time over which humic acid stimulation continues increases with the concentration; and that, as we have shown elsewhere (2, 3, 4), (*a*) humic acid stimulates cultures grown in free or fixed nitrogen, (*b*) the stimulation is decreased as the oxygen pressure is decreased below 0.21 atmosphere, (*c*) the efficiency of growth increases as the rate of growth is stimulated by humic acid, (*d*) humic acid is heat-stabile at autoclaving temperatures. Iwasaki's work is therefore in substantial agreement with our work wherever the same phenomena have been investigated; it is less extensive, however, and subject to the disadvantage that velocity constants of growth, i.e., logarithmic functions of growth, were not employed. On this latter account, and because of greater experimental error, Iwasaki was unable to observe, as we have in figure 7, that stimulation as represented by an increase in the velocity constant of growth is a definite function of concentration of humic acid.

Induction in humic acid substitution compounds

Various natural humic acids present minor differences in induction length, and consequently the same differences should be expected between various synthetic iron-containing humic acids or between the latter and natural ones. Table 10 illustrates a few experiments comparing the synthetic and natural humic acids, VII and V, in which it is seen that roughly the same induction period obtains in both cases. In the light of all our work on this subject, it can be said that, in general, synthetic acids can be prepared to yield the same induction period, viz., a few hours, as the natural humic acids, but that sometimes a given synthetic humic acid will show a distinctly longer induction period.

As a result of gathering together a considerable number of pertinent experiments similar in import to those in table 9, it may be said that organic acid iron compounds of the type of ferric citrate show an induction period of generally 8 to 15 hours (very occasionally less, however, or even more), whereas with inorganic iron compounds the induction is generally 15 to 30 hours.[3]

The Influence of Humic Acid upon the Oxidation-Reduction Potential of the Medium

Studies carried out during the last decade, and in particular during the last year or two [Knight (12), Hewitt (10), and Allyn and Baldwin (1)] have indicated that the oxidation-reduction (O/R) potential of the medium may influence markedly the growth of bacteria, and *vice versa*. In view of the

[3] It is not difficult to understand, on grounds of solubility alone, why inorganic iron should show much the greatest induction phase. In stating in the various experiments that inorganic iron of a certain concentration is added, no implication is intended that any considerable fraction remains in solution. Thus an addition of 2 p.p.m. Fe as $Fe_2(SO_4)_3$ would yield about 0.01 p.p.m. soluble Fe. The initial soluble iron content of customary medium containing 1 per cent glucose is considerably higher (about 0.035 to 0.057 p.p.m.). Since the minimum equilibrium concentration of iron of any type yielding optimum stimulation of growth is about 0.5 p.p.m., *inorganic iron must almost always be limiting, and will compare favorably with humic acid iron (etc.) only when the latter is supplied in similarly limiting concentrations.* This point cannot be too strongly emphasized. Upon this basis it is easy to understand the universal observation that the ash of humic acid, containing iron, is usually relatively inactive compared to the original sample of unashed humic acid. On the other hand, all humic acid and organic acid iron is soluble. Since the induction phase is still slightly greater in the latter case than in the former case it is evident that solubility is not the sole physiological factor governing differences in induction-time values between different iron-compound types. The fact that the iron in humic acid is partly in an adsorbed condition very probably explains its greater availability compared to iron bound to organic acids, which is in chiefly a non-ionic complex form. Finally, it is to be pointed out that although the simple organic acid and inorganic iron compounds show in general greater periods of induction than natural (or synthetic) humic acids, the fact that they show induction at all is in itself additional evidence in favor of the view that iron is the cause of humic acid stimulation, because of the qualitative similarity to the humic acids; since the simple iron compounds obviously possess secondary chemical differences they can be expected to possess secondary physiological differences.

proved connection of humic acid stimulation with iron (8), and the relation of iron, even in small concentrations, to systems of interest in connection with O/R potentials, it is obviously pertinent to determine whether humic acid stimulation is related to any change which humic acid may impose upon the O/R potential of the medium.

We have employed the La Motte series of dyes: 1. o-chlorophenol indophenol, 2. phenol indophenol, 3. o-cresol indophenol, 4. l-napthol-2-sulfonate indophenol, 5. methylene blue, 6. indigo tetrasulfonate, 7. indigo disulfonate, and 8. indigo monosulfonate, with respective E_o values at pH 7.0 of 0.233, 0.229, 0.195, 0.119, 0.011, −0.046, −0.125, and −0.157. The cultures have always been adjusted initially to pH 7.0, by the appropriate and necessary addition of either mono- or di-potassium phosphate. The *Warburg* technique has been used for experiments conducted in an oxygen-free atmosphere, and both the *Warburg* and *Erlenmeyer* techniques for cultures maintained in air. In the anaerobic experiments care was taken to have removed the last traces

TABLE 12

The influence of hydrogen as compared to nitrogen upon the O/R potential under anaerobic conditions (Warburg technique)

	GAS					
	N_2	H_2	N_2	H_2	N_2	H_2
20 p.p.m. dye	2	2	5	5	7	7
Per cent reduction, 5 minutes	40	40	..	..	..	..
Per cent reduction, 18 minutes	..	..	50	50	..	..
Per cent reduction, 180 minutes	..	..	..	..	40	40

Three-day-old *A. vinelandii*. 31°C. Dyes tipped into cultures from side-cups of respiration vessels after last traces of oxygen removed as shown by zero respiration readings (KOH in the well). Experiments performed in duplicate.

of oxygen before adding the dye to the medium. In general the colorimetric dye comparison measurements were accurate to ± 5 per cent.

Call suspensions of Azotobacter, in common with those of many other bacteria [Cannan, Cohen, and Clark (9)], are poorly poised with respect to the electromotively active material present at any moment. With cultures at least 1 day old, the percentage rates of reduction are relatively independent of concentration of dye up to 100–200–500 p.p.m. In air, a positive O/R potential of about +0.20 volt is assumed, as indicated by partial reduction (in general 50 per cent, see table 14) of dye 3, attained after one-half to several hours. The rate depends a good deal upon the concentration of dye employed and the extent of shaking employed to maintain equilibrium with the 21 per cent O_2 in the air which the cultures through respiration tend to lower. Dye 2 is reduced completely and dye 4 is not reduced at all. In N_2, a negative potential of about −0.15 v. is assumed after some hours of anaerobiosis (see table 13). Dyes 1, 2, 3 (at concentrations of 5–100 p.p.m.) are reduced 95 per cent in about 15 minutes, dye 4,95 per cent in about 30 minutes, dye 5, 95 per cent in 1 to 2 hours, and dye 7, 95 per cent in 6–12 hours. Reduction of dye 8 has never been observed to an extent greater than 5 ± 5 per cent. Upon admission of air to, say 90–95 per cent reduced dye 7 (in anaerobic

Azotobacter cultures), the oxidized form (0–10 per cent reduced) is assumed in a few seconds, and to other more positive dyes correspondingly longer times (1 to 15 minutes); with dyes 1 and 2 little or no reoxidation is obtained. In H_2 the same results are obtained as in nitrogen

TABLE 13

The influence of natural and synthetic humic acids upon the O/R potential of cultures of customary medium maintained in pure N_2 (Warburg technique)

Experiment 1:												
P.p.m. dye 7..	4	8	30	90	15	15	15	15	15	15	15	15
P.p.m. H. A...	–	–	–	–	–	–	V–10	V–150	VI–50	IX–50	X–50	XVIII–50
Per cent reduction, 5 hours.....	90	65	65	70	80	75	85	60	75	55	55	65
Experiment 2:									(Dye 8			
P.p.m. dye 7..	10	10	40	40	100	100	200	200	10)	..	...	...
40 p.p.m. H. A.-V.....	+	–	+	–	+	–	+	–	–	..	...	...
Per cent reduction, 11 hours.....	..	..	95	95	85	85	...	...	0–5	..	...	...
Per cent reduction, 17 hours.....	95	95	95	95	95	95	75	75	..	..	...	...
Per cent reduction, 21 hours.....	..	..	..	..	..	..	85	85	..	..	...	...
Experiment 3:												
P.p.m. dye 7..	20	20	20	20	20	20	20	20	5	5	100	100
P.p.m. H. A.-V.....	5	–	20	–	40	–	150	–	40	–	40	–
Per cent reduction, 17 hours.....	0	0	0	0	0	0	0	0	0	0	0	0
Experiment 4:												
50 p.p.m. dye.	3	3	3	4	4	4	...	..	..	..	...	...
10 p.p.m. dye.	..	..	..	..	..	..	5	5	5	..	...	...
P.p.m. H. A....	–	V	VI	–	V	VI	–	V	VI	..	...	...
Per cent reduction, 7 hours.....	0	0	0	0	0	0	0	0	0	..	...	...

Experiment 1: 3-day-old *A. vinelandii*. 31°C.

Experiment 2: 2-day-old *A. vinelandii*. 31°C.

Experiment 3: Customary medium containing 1 per cent glucose (no Azotobacter). About 23°C.

Experiment 4: Customary medium containing 1 per cent glucose (no Azotobacter). 31°C.

Experiments performed in duplicate.

(see, for example, table 12). The experiments with hydrogen, exampled by table 12, are important in showing, even more delicately than hitherto (2), that Azotobacter is unable to consume hydrogen gas, i.e., hydrogen is completely inert, and that Azotobacter possesses no

hydrogenase similar to that recently discovered by Stephenson and Strickland (16) capable of activating hydrogen gas to reduce oxygen gas, methylene blue, nitrate and fumarate, etc. The medium in the absence of Azotobacter ("customary" medium containing 1 per cent glucose) has no detectable effect on dye reduction either aerobic or anaerobic (see table 13), under the various experimental conditions employed. In short-time, aerobic, *Warburg* technique experiments, no beneficial effect of dye on growth (respiration rate increase) was observed, when 10 and 100 p.p.m. dye 2, 5 and 50 p.p.m. dye 5, and 5, 25, and 100 p.p.m. dye 7 were used; whereas in the same experiments, H. A.-V exerted a stimulating effect by the second hour and had established by the sixth hour a humic acid ratio of 1.5. O/R effects are substantially independent of the age of culture. In one experiment, in N_2, using 1, 3, 7, 10, 12, 13, and 38-day-old cultures, 75–90 per cent reduction of 20 p.p.m. of dye 7, at about 23°C., was obtained in 8 hours and remained thus for 30 hours. In another experiment, in air, using 1,

TABLE 14

The influence of natural and synthetic humic acids upon the O/R potential of cultures or customary medium maintained in air (Erlenmeyer technique)

Experiment 1:								
20 p.p.m. dye	3	3	3	3	4	4	4	4
P.p.m. H. A.-V	0	20	50	100	0	20	50	100
Per cent reduction, 1 hour	40	40	40	40	0	0	0	0
Per cent reduction, 2 hours	40	40	40	40	0	0	0	0
Experiment 2:								
P.p.m. dye 3	50	50	150	150	.	..	..	...
50 p.p.m. H. A.-V	50	..	50	...	.	..	..	...
Per cent reduction, ½ hour	35	35	15	15	.	..	..	...
Per cent reduction, 1½ hour	35	35	15	15	.	..	..	...
Experiment 3:								
P.p.m. dye 3	50	50	50	50	.	..	..	...
50 p.p.m. H. A.	..	V	VI	X	.	..	..	...
Per cent reduction, 20 minutes	80	80	80	80	.	..	..	...
Experiment 4:								
P.p.m. dye 3	20	20	20	...	.	..	..	...
25 p.p.m. H. A.	..	V	X	...	.	..	..	...
Per cent reduction, 5 hours	50	50	50	...	.	..	..	...
Per cent reduction, 24 hours	50	50	50	...	.	..	..	...

Experiments 1, 2, 3, 4: 3, 3, 17, 5-day-old *A. vinelandii*. All experiments in duplicate except No. 3, in triplicate, temperature about 23°C.

2, 5, 7, 14, 19, 20, and 44-day-old cultures, 20–90 per cent reduction of 50 p.p.m. of dye 3 at about 23°C. was obtained in ½ to 3 hours, and maintained thereafter indefinitely. The younger 1- and 2-day-old cultures required somewhat the longer reduction times, and in general, with very young cultures one must employ relatively smaller concentrations of dye to obtain equal reduction times as compared with cultures 2 days old or more; the O/R capacity factor is undoubtedly smaller in very young cultures. The effect of oxygen tension upon O/R potential of cultures is easily observed. In one particular experiment with 5-day-old *A. vinelandii* maintained in *Warburg* vessels at 100, 21, and 2 per cent O_2 (in N_2) the per cent reductions of 50 p.p.m. of dye 3 obtained after 3 hours were 20, 50, and 90 per cent, respectively (corresponding to a maximum difference of about 0.05 volt). It is highly probable that the unique oxygen pressure functions of growth, respiration, and growth efficiency (3) are in some degree related to O/R potentials established at different oxygen pressures.

As seen in tables 13 and 14, humic acid exerts no measurable effect upon the O/R potential of either cultures of *Azotobacter* or the customary medium maintained under either aerobic or anaerobic conditions. This is true for both natural humic acids (H. A.-V) and synthetic humic acids, independently of whether the latter are iron-rich (viz., H. A.-IX, X) or iron-poor (viz., H. A.-VI, XVIII). Moreover, the rates of dye reduction are likewise completely unaffected. Concentrations of humic acid varying from 4 to 150 p.p.m. have been employed, and the dyes have varied in concentration from 5 to 200 p.p.m. Although humic acid might exert an O/R potential of exceedingly small magnitude, possibly detectable by electrometric technique, it is certain from these results that, so far as growth of Azotobacter cultures is concerned, no significant influence is involved.

Does Humic Acid Act by Increasing Directly the Availability of the Known Constituents and Physical Properties of the Medium?

Tables 15 to 19 show that the humic acid ratio is on the whole independent of the nature of the inorganic medium in experiments of ordinary duration. The variations between the limits 1.4 and 4.5 are quite random, and can be assigned consistently to no particular inorganic causes. The independence is particularly striking, as shown in experiment 7, table 15, and in the case of the first two media employed in table 17; on the one hand, all the customarily added ions are present, and, on the other hand, the only other inorganic ion present is Na (except NO_3 and HCO_3, but these are present in both cases as a result of addition or of production in respiration). Tables 16 and 17 show particularly that the humic acid ratio is not significantly affected by either the presence or the absence of Ca, Mg, Fe, K, Na, Cl, PO_4, or SO_4, under the given conditions. Table 18 shows that the inoculum employed in tables 15, 16, and 17 can not have supplied sufficient of the customary inorganic salts to suffice for one generation or transfer, since after seven transfers the ratio has remained unchanged, within errors of experimental measurement. Table 19 eliminates, as far as possible, the likelihood of these customary salts being supplied in either the glucose or the bicarbonate employed and a number of other similar experiments, not reported, gave the same results also.

Table 20 shows that humic acid has no immediate and direct effect upon CO_2 production, glycolysis (the formation of lactic acid, or some other acids), glucose consumption, or O_2 consumption. The date of table 11, and also the data of table 19, as compared with that of table 18, in some measure offer additional confirmation with respect to glucose consumption, since the initial glucose concentration has been varied 10-fold. In several hundred experiments with the *Warburg* technique, where cultures have been maintained in the presence or absence of humic acid, it is ordinarily impossible to detect any appreciable increase (3 to 5 per cent) in respiration during the first hour after the addition of the humic acid, whereas, on the other hand, common stimulants or depressants of respiration, such as changes in O_2 tension, HCN, and in-

different narcotics, almost invariably exert their complete effect within a few minutes.

The humic acid ratio does not depend upon the source of nitrogen available for growth, provided that the growth velocities in free and in the various forms

TABLE 15

Miscellaneous experiments on the magnitude of the humic acid ratio in the presence and absence of fixed nitrogen

EXPERIMENT NUMBER	MEDIUM	FINAL NET TURBIDITY (RELATIVE GROWTH)		H. A.-RATIO
		H. A.-I (25 p.p.m.)	No humic acid	
Experiment 1 (a):				
a. In N_2	Customary	450	100	4.5
b. In NO_3	Customary	982	295	3.3
Experiment 2 (a):				
a. In N_2	Customary	371	158	2.4
b. In NO_3	Customary	982	340	2.9
Experiment 3 (a):				
a. In N_2	Customary	522	143	3.6
b. In NO_3	Customary	886	384	2.3
Experiment 4 (a):				
a. In N_2	Customary	458	109	4.2
b. In NO_3	Customary	833	384	2.2
Experiment 5 (b):				
a. In N_2	Customary	99	30	3.3
Experiment 6 (c):				
a. In N_2	Customary	60	41	1.5
b. In NO_3	Customary	162	98	1.6
Experiment 7 (d):				
a. In NO_3	Customary	117	50	2.3
b. In NO_3	Bicarbonate	18	5	3.6
Experiment 8 (e):				
a. In NO_3	Customary	207	85.8	2.4
Experiment 9 (e):				
a. In NO_3	Bicarbonate	186	103	1.8
Experiment 10 (e):				
a. In NO_3	Customary	287	174	1.7

a, b, c, d, e, inoculated with 2 drops 3-day, 1 drop 2-day, 1 drop 4-day, 1 drop 2-day, 1 drop 2-day-old *A. vinelandii*; analyzed after 48, 41, 74, 52, 48 hours. The relative values are relative only within any given experiment, and not between different experiments.

In experiments 1, 3, and 4, specifically, and many other similar experiments, the humic acid ratio is lower in NO_3-N than in N_2-N under otherwise comparable conditions, undoubtedly because growth in NO_3-N in humic acid is approaching its ultimate value under the given conditions and the velocity of growth is decreasing relatively more rapidly.

of fixed nitrogen in the absence of humic acid are approximately equal [table 15; (8); tables 3A, 11A; (11); (2); (3); (4); (5)].

It does not seem probable that humic acid exerts its effect by virtue of

TABLE 16

The magnitude of the humic acid ratio in bicarbonate medium with different salts added

COLUMN 1	COLUMN 2	COLUMN 3	COLUMN 4	COLUMN 5	COLUMN 6	COLUMN 7	COLUMN 8

TABLE 16

The magnitude of the humic acid ratio in bicarbonate medium with different salts added

Bicarbonate medium plus	COLUMN 1 Nothing		COLUMN 2 ½ customary $CaSO_4$		COLUMN 3 1/10 customary $CaSO_4$		COLUMN 4 Customary $Fe_2(SO_4)_3$		COLUMN 5 1/10 customary $Fe_2(SO_4)_3$		COLUMN 6 Customary NaCl		COLUMN 7 Customary $MgSO_4$		COLUMN 8 Customary K_2HPO_4 / KH_2PO_4	
H. A.-I (25 p.p.m.)	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Final gross turbidity	102	32.2	65.3	31.3	79.5	22.9	104	70.0	94	39.4	91.0	26.7	110	69.8	115	67.0
	98	35.3	64.1	29.2	74.6	33.2	146	74.4	93	48.3	95.5	26.9	107	66.8	.....	51.2
Turbidity uninoculated control	5.8	2.3	12.7	10.9	4.9	2.5	36.7	27.4	10.4	7.4	5.8	2.9	3.8	2.3	3.9	2.3
Final average net turbidity = growth	94.2	31.5	52.3	19.3	72.2	25.5	88.3	44.8	83.1	36.5	87.5	23.9	105	66	111	56.8
H. A.-ratio	3.0	.....	2.7	.....	2.8	.....	2.0	.....	2.3	.....	3.7	.....	1.6	.....	1.9	.....

Inoculated with 1 drop of 2-day-old *A. vinelandii*, examined after 48 hours. Final pH in all flasks 7.5. Source of nitrogen: 50 p.p.m. NO_3-N. VI/21–23/30. Volumes of culture solutions 34 cc., not 25. cc., as ordinarily.

In this experiment the sugar was added after sterilization (as sterile glucose) rather than before sterilization, as usual in other experiments. Bicarbonate medium turns somewhat brown when sterilized with glucose, no doubt, because the pH is 7.5, which accelerates decomposition.

TABLE 17

The magnitude of the humic acid ratio in media of different inorganic compositions

	MEDIA																	
	Customary		$NaHCO_3$*		$KHCO_3$		$KHCO_3$ plus $CaSO_4$†		$KHCO_3$ plus $MgSO_4$†		$KHCO_3$ plus NaCl†		$KHCO_3$ plus $Fe_2(SO_4)_3$‡		$KHCO_3$ plus $CaSO_4$‡		$KHCO_3$ plus $Fe_2(SO_4)_3$†	
H. A.-I (25 p.p.m.)	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Final net turbidity = growth	115¶	72.5¶	31.2	21.2	47.6¶	20.3¶	20.2	10.4§	22.3	9.5§	39.5	21.5	46.5	22.2	32.1	16.0	Lost	19.1
H. A.-ratio‖	1.6	....	1.5	....	2.3	....	2.0	.....	2.3	....	1.8	....	2.1	....	2.0	....	−	....
Final pH	6.8	6.8	7.2	7.2	7.2	7.2	6.9	6.9	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	..	7.2

Inoculated with 1 drop of 2-day-old *A. vinelandii*. Analyzed after 72 hours. Source of nitrogen: 50 p.p.m. NO_3-N present in all cultures as KNO_3, except in $NaHCO_3$ cultures when present as $NaNO_3$. VI/4–7/30.

* NO_3 added as $NaNO_3$.

† At customary concentration.

‡ At one-tenth customary concentration.

§ The depressing effect of $CaSO_4$ and $MgSO_4$ in bicarbonate medium in both the presence and absence of humic acid is to be noted. This occasionally takes place in nitrate cultures (never in N_2 cultures).

¶ It is to be observed that the rate of growth in customary medium to that in bicarbonate medium is here 115/47.6 = 2.4 in the presence of humic acid, and 72.5/20.3 = 3.6 in the absence of humic acid.

‖ It is evident from this experiment and that reported in table 16 that the humic acid ratio is not *significantly* affected by the presence or absence of intentionally added Ca, Mg, Fe, K, Na, Cl, SO_4.

deactivating metabolically produced toxic products, since it has already been found [(5), and much unpublished data] that, under our various experimental conditions at least, Azotobacter does not produce during its metabolism (for cultures up to 30 days old) any metabolic products which are toxic to either respiration, growth, or the chemical mechanism of nitrogen fixation.

Direct measurements with capillary tubes show that concentrations of 25 to 50 p.p.m. of natural humic acid change the viscosity and the surface tension

TABLE 18

The humic acid ratio as influenced by repeated transfer in bicarbonate medium

	MEDIUM			
	Bicarbonate		Customary	H. A.-ratio
	H. A.-I (25 p.p.m.)	No humic acid		
Solution designation	A	B	C	A/B
Transfer 1	100	33	400	...
Transfer 2	68.4, 74.4 } 71.4	33.2, 33.4 } 33.3	331, 309 } 320	2.1
Transfer 3	84.2, 77.2 } 80.8	38.9, 39.7 } 39.3	610, 518 } 564	2.1
Transfer 4	124	54	443	2.3
Transfer 5	101, 89 } 95	51.4, 50.6 } 51.0	307, 339 } 323	1.9
Transfer 6	82, 88.7 } 85.4	58.8, 55.6 } 57.2	346, 299 } 323	1.5
Transfer 7	103, 90 } 97	49	450	2.0

Transfer 1 inoculated with 1 drop of 1-day-old *A. vinelandii*; transfer 2 inoculated with 1 drop of transfer 1, solution B, after 2 days' growth. Transfer 3 inoculated with 1 drop of transfer 2, solution B, after 2 days' growth, and so on—in each case the fresh transfer was made from the one of the bicarbonate medium-without-humic acid flasks just before analysis. Final pH: Solutions A and B, 7.5; solution C, 7.1. Source of nitrogen: 50 p.p.m. NO_3-N. III/–/30.

The growths in solution C are given to indicate the rate of growth in the bicarbonate medium as compared with that in the customary medium, and also to show that the organisms in solution B would, at any transfer, when inoculated in solution C, give approximately normal growth.

All growth values for every transfer are strictly relative to each other and to those in table 19.

of the customary medium by no more than a few per cent (i.e., within experimental error of measurement), if at all. Measurements of the influence of humic acid upon the potential difference (P. D.) between culture medium and organism have not been made, but a significant influence is reasonably precluded when it is considered that some preparations of humic acid are effective at concentrations as low as 1 to 3 p.p.m. It would seem probable, also, that

in the experiments reported in tables 15–19, and others, the potential difference (P. D.) varied considerably because of the great differences in salt concentration.

Summarizing the data of this Section, we may point out that all the evidence indicates that humic acid does not improve any of the known physical or

TABLE 19

The humic acid ratio in 10-fold diluted bicarbonate medium

	MEDIUM			
	10-fold diluted	Bicarbonate medium*	Customary	H. A.-ratio
	H. A.	No H. A.	No H. A.	
Transfer 6†	105, 108 } 107	44.5	195, 203 } 197‡	2.4

* The medium consisted of 0.1 the customary amounts of glucose and potassium bicarbonate, i.e., 0.1 per cent glucose, and 0.01 per cent (= 100 p.p.m.) $KHCO_3$. This was done in an attempt to eliminate possible impurities in these substances whose availability the humic acid might be increasing. Source of nitrogen: 50 p.p.m. NO_3N (recrystallized). III/–/30.

† The cultures were inoculated with the same inoculum used in inoculating transfer 6, table 18, i.e., from 5B. Analyzed after 48 hours. Growth values relative to those of table 22. Final pH: 7.3, except in customary medium, 7.0.

‡ This value is lower than those of table 18, because no doubt the glucose concentration has become limiting by the time a relative growth of 150–200 is reached.

TABLE 20

The influence of humic acid upon the production of weak acids during respiration

	COLUMN 1. KOH ABSENT (CMM. RESPIRED CO_2 DISSOLVED IN MEDIUM*)	COLUMN 2. KOH PRESENT (CMM. O_2 CONSUMED)
Experiment 1:†		
N_2	26.5, 25.4, 25.9, aver. 25.9	146, 149, 152, aver. 149
N_2 plus 50 p.p.m. H. A.-II	26.8, 25.4, 25.6, aver. 25.9	150, 151, 146, aver. 149
Experiment 2:‡		
N_2	14.4, 12.0, aver. 13.2	50.7, 50.3, aver. 50.5
N_2 plus 25 p.p.m. H. A.-II	13.5, 12.6, aver. 13.1	54.2, 45.2, aver. 49.5

* This value is directly proportional to the total respired CO_2.

† Two-day-old *A. vinelandii*. 2 cc. culture per vessel employed. Duration of experiment 60 minutes. Humic acid had been added to medium 30 minutes before readings taken. Gas phase: 21 per cent O_2 in N_2. Final pH, column 1, 6.6 ± 0.1; column 2, 6.8±0.1. VIII/-30/30.

‡ 1-day-old *A. vinelandii*. 1 cc. culture per vessel employed. Duration of experiment 12 hours. Gas phase: 1 per cent O_2 in N_2. Final pH, column 1, 6.6 ± 0.1; column 2, 7.0 ± 0.1. VIII/29/30.

chemical properties of various widely different media. This conclusion substantiates the findings of the previous paper (8) that humic acid exerts its effect by *adding* something to media, namely, iron.

SUMMARY

This paper presents various functional relationships obtaining when the growth of Azotobacter is stimulated by humic acid or iron compounds. Particular care is taken to distinguish those functions which depend upon time from those which do not. Frequent use is made of the velocity constant of growth, rather than the velocity of growth, in determining the stimulation functions.

Humic acid stimulation of Azotobacter growth involves an induction period of several hours, during which time no stimulation takes place. The induction period is substantially independent of temperature, source of nitrogen, glucose concentration, humic acid preparation, concentration of humic acid, age of culture, heaviness of culture, respiration rate, moment of addition, the growth velocity in the absence of humic acid, and the magnitude of the subsequent stimulation. Synthetic iron-containing humic acids may possess the same induction period as natural humic acids, but organic acid iron compounds and inorganic iron compounds possess definitely longer induction periods.

The induction period is sharply defined; when stimulation commences a new velocity constant of growth is assumed within about one hour, the value of the constant depending, among other factors, upon the concentration of humic acid.

Stimulation increase in growth velocity constant attains a maximum at about 50 p.p.m. humic acid, or about 0.5 p.p.m. iron, which is the same iron concentration as obtains with respect to the three other iron compound types: synthetic iron-containing humic acid, organic acid iron, and inorganic iron. As the active fraction of the stimulant is consumed, during growth, the initial concentration giving maximum growth may be increased to 5 to 15 p.p.m. iron, corresponding to about 500 to 1,500 p.p.m. natural humic acid.

At constant temperature all factors observed to cause increased velocity constants of growth caused both relative and absolute decreased stimulation, with respect to nitrogen source, pressure of nitrogen gas, concentration of fixed nitrogen, medium constitution, age of culture, heaviness of culture, glucose concentration, and oxygen pressure. When the velocity constant of growth is increased as a function of sub-optimal temperatures, the converse relationship obtains, i.e., stimulation is increased.

Natural and synthetic humic acids exert no significant influence upon the oxidation-reduction character of either cultures of Azotobacter or the culture medium in the absence of organisms, both with respect to rates of potential change and ultimate potentials assumed. The cause of humic acid stimulation is consequently independent of oxidation-reduction potential factors. In this connection, the oxidation-reduction character of Azotobacter cultures in the absence of humic acid has been described; under aerobic and anaerobic conditions, potentials of about $+0.20$ and -0.15 volts, respectively, are assumed. Oxidation-reduction dyes have not been observed to stimulate growth under conditions where humic acid stimulation takes place.

Humic acid does not act by increasing directly the availability of constituents normally added to, or present in, the medium, viz., glucose, oxygen, free or fixed nitrogen, carbon dioxide, Ca, Mg, Na, K, Fe, PO_4, Cl, SO_4, HCO_3; or by deactivating toxic metabolic products; or by affecting the surface tension, viscosity, or potential difference between culture medium and organism.

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BOOK REVIEWS

Literatursammlung aus dem Gesamtgebiet der Agrikulturchemie, vol. I. Bodenkunde. By H. NIKLAS, F. CZIBULKA AND A. HOCK. Edited by H. NIKLAS and A. HOCK. Verlag des Agrikulturchemischen Instituts Weihenstephan der Technischen Hochscule, München, Germany, 1931. Pp. xxxvi + 1008.

A century of research in the field of soil science has created an imposing mass of information in many languages, scattered through periodicals, monographs, dissertations, and reports. Most of the information of interest to students of soils and plants has been gathered and published since the beginning of the present century. He who would keep abreast of the times must be more or less familiar, not alone with the scientific literature in his chosen field, but in contiguous fields. To this end the authors have made a notable contribution.

There are eight sections in this volume. The first contains a reference to literature of a more or less general character. The second section—comprising nearly 200 pages—has to do with the formation of soils. The fields of geology, mineralogy, physics, chemistry, and climate are all represented in this group of references. In the third section—pages 217 to 408—there are given the titles of papers dealing specifically with soil chemistry. Similarly, section four—pages 411 to 590—is devoted to soil physics; section five—pages 590–767—, to the influence of external factors on soils; section six—pages 773 to 789—, to soil inventories; section seven—pages 801–911—to soils of different origin; and section eight—pages 915 to 939,—to the composition of soils, rocks, and minerals.

For the convenience of the reader who does not possess a ready command of German, the preface, introduction, and table of contents are given also in English. Throughout the body of the book, the general topics are given in both German and English. For instance, on page 69, the reader will find the general topic "Auswaschung—Leaching.": on page 130 is given "Tropische und subtropische Böden" and its English equivalent "Tropical and Subtropical Soils." In each instance the reference consists of the name of the author, or authors, and the title of the paper—the latter usually in German or English. Relatively few of the titles are given in the original where languages other than German or English are concerned.

The extent of the field covered is readily indicated by the approximately 20,000 titles appearing in this volume. As is noted by the authors, "The bibliographical collection—in the way we have accomplished it,—is not able to comprehend all corresponding work of the various countries. We neither intended nor were able to do so. On the other hand all competent work is mentioned that was discussed in one of the 18 central journals, respectively central organs previously referred to." They say further: "Our work offers

most serviceable and extensive rudiments, and may in a large measure help to start in all other scientific fields in the same way as we did, the absolutely indispensable collection of literature, that hitherto from every point of view seems to be excessively disunited."

Literatursammlung aus dem Gesamtgebiet der Agriculturchemie, vol. II. Boden Untersuchung. By H. NIKLAS, F. CZIBULKA, AND A. HOCK. Edited by H. NIKLAS and A. HOCK. Verlag des Agrikulturchemischen Instituts Weihenstephan der Technischen Hockschule, München, Germany, 1931. Pp. xxviii + 199.

The organization of the references given in this volume is identical with that adopted for volume I of the series. The authors note that "The publishing of a special volume II 'Bibliographical List of the Entire Domain of Soil Analysis' was intended particularly on the one hand to put the importance of this subject,—that in the last decade has been thoroughly elaborated by scientists of various countries—to good account, and on the other hand to meet the wishes of all those, who work exclusively at the analysis of the soil, but on the other hand not in general at the science of soil. For in the course of the last years certainly the scientific field of 'Soil Analysis' has become an independent branch of science—complete in itself—that according to its whole condition can neither be completely arranged in the sphere of 'Soil Science' nor in the sphere of 'Plant Nutrition.' "

The field covered in this book on the methodology of soil investigations includes the major topics of sampling, chemical analysis, physical analysis, and microbiological analysis. Some space is devoted also to microscopic soil analysis, mineralogical soil analysis, and analysis of rocks. As in the first volume of the series, there is a subject index and an author index. Like the first volume, it will serve as an extremely useful—in fact, indispensable—reference book. It should be made readily available to every worker in the field of soil science and plant nutrition.

Man and Microbes. By STANHOPE BAYNE-JONES. The Williams & Wilkins Company, Baltimore, Md., and Associates in Cooperation with The Century of Progress Exposition, 1932. Pp. X + 128, figs. 16.

The commemoration of the 100th anniversary of the founding of the City of Chicago has paved the way for various enterprises of an educational character. One of these is The Century of Progress Exposition itself. In conjunction with the organization of the exposition, provision has been made for the publication of pamphlets, monographs, and books as a means of bringing about a better understanding of the contributions that science has made to the development of our present economic and social structure. As a popular treatise on the significance of microbes to human society, this book should find favor with the many busy folks who wish to keep in step with the progress of applied science.

The author tells us that "In this small book, I have tried to give the microbes their due, indicating their useful and essential activities as well as their devastating effects. The occasional spectacular havoc wrought by the germs of disease should not divert attention from the invaluable chemical services which hosts of microbes are quietly rendering man, animals and plants all the time."

The nine chapters of the book deal respectively with Microbes; Microbes and the Soil; Microbes in Air, Water and Sewage; Microbes in Industry; Microbes and Plants; Microbes and Insects; Microbes and Diseases of Animals Transmissible to Man; Microbes and Diseases of Man, and Microbes and Civilization.

The Waste Products of Agriculture. Their Utilization as Humus. By ALBERT HOWARD AND YESHWANT D. Wad. Humphrey Milford, Oxford University Press, London, New York, Toronto, Melbourne, Bombay, Calcutta, Madras, 1931. Pp. xiv + 167, plates 14, figs. 9.

The authors' understanding of life and conditions in the Far East have undoubtedly influenced their approach to the subject dealt with in this book. They say, in the preface: "One of the main features of crop production at the present day is waste. Except in the Far East, where the large indigenous population has to be fed from the produce of the country-side, little is being done to utilize completely the by-products of the farm in maintaining the fertility of the soil. The ever-growing supplies of agricultural produce, needed by industry and trade, have been provided either by taking up new land or by the purchase of artificial manures. Both these methods are uneconomic. The exploitation of virgin soil is a form of plunder. Any expenditure on fertilizers which can be avoided raises the cost of production, and therefore reduces the margin of profit. It needs no argument to urge that, in maintaining the fertility of the soil, the most careful attention should be paid to the utilization of the waste products of agriculture itself before any demands are made on capital—natural or acquired."

The six sections of the book consist of Introduction; Organic Matter and Soil Fertility; The Sources of Organic Matter; The Manufacture of Compost by the Indore Method; The Chief Factors in the Indore process; and Application to Other Areas. There are also four appendixes dealing with The Manurial Problem in India; Some Aspects of Soil Improvement in Relation to Crop Production; Nitrogen Transformation in the Decomposition of Natural Organic Materials at Different Stages of Growth; and An Experiment in the Management of Indian Labour.

To the student of the Far East this volume will prove both interesting and useful for it has to do with some of the basic factors in the life of the teeming millions of India. The fundamental principles which are followed in extracting human and animal food from ancient soils are an expression of age-long experience. Science is now beginning to touch the practice of the tropics.

The authors are to be commended for having made a distinct contribution to an important phase of soil fertility.

Arbeiten über Kalidüngung. By O. Eckstein, A. Jacob, and F. Alten. Verlagsgesellschaft für Ackerbau M. B. H., Berlin, 1931. Pp. 237, figs. 72.

The German Kalisyndikat established in 1929 an experiment station at Berlin-Lichterfelde. It was provided in the research program of the founders that the activities of the station deal with soil and plant nutrition problems not alone in Germany, but in various soil regions of temperate, subtropical, and tropical countries. This book constitutes a report on the present status of the research activities of the station.

Four general topics are dealt with by the authors; namely, objectives of an experiment station specifically dealing with potash research; a discussion of the physical plant and resources of the station; soil profiles and mapping of the land used by the experiment station at Berlin-Lichterfelde; and projects and results of the station. In the last division, we find the following: 1, Laboratory studies of soils with special consideration of tropical soils; 2, Comparative tests of different methods for the determination of soil potash readily assimilable by plants; 3, Studies of the content of potash in plants and its chemical combinations; 4, The influence of fertilization with potash on the composition of crop products; 5, Potash salts as protective agents of plants; 6, The physiological reaction of potash salts; 7, The fertilizer action of anions and other constituents of potash salts; 8, Tests of the fertilizer action of different materials containing potash and magnesia; 9, Studies on the compatability in mixtures of different commercial fertilizers; and 10, Investigations on the significance of potash for the animal organism.

The Microscopic Characters of Artificial Inorganic Solid Substances or Artificial Minerals, second edition. By Alexander Newton Winchell. With a Chapter on The Universal Stage by Richard Conrad Emmons. John Wiley & Sons, Inc., New York, and Chapman & Hall, Ltd., London, 1931. Pp. xvii + 403, figs. 311.

This is the second edition of a book that has evidently made a useful place for itself among recent text and reference books. The scope of the field treated by the author is indicated by "Principles and Methods" of Part I and "Descriptions of Artificial Inorganic Solid Substances" of Part II. There are, aside from the introduction, eight chapters in Part I, dealing with Crystals; Apparatus and Preparation of Materials; Optic Properties of Isotropic Substances; Optic Properties of Uniaxial Crystals; Uniaxial Crystals in Parallel Polarized Light; Optic Properties of Biaxial Crystals; Biaxial Crystals in Parallel Polarized Light; and The Universal Stage. The eleven chapters of Part II include: Elements; Sulphides, Etc.; Halides and Cyanides; Cyanates and Thiocyanates; Oxides; Carbonates, Etc.; Borates, Etc.; Sulphates, Etc.; Phosphates, Etc.; Columbates, Tantalates, Titanates and Zirconates; and

Silicates. The third and last part of the book, entitled "Determinative Tables," includes Table I, Isotropic Artificial Solid Substances; Table II, Anisotropic Artificial Inorganic Solid Substances; and Index. The illustrations and general workmanship are in keeping with the excellence of the standards maintained by the publishers.

Report for 1930 of the Rothamsted Experimental Station, Harpenden, Herts, England. By SIR E. JOHN RUSSELL, Director. Gibbs & Bamforth, Ltd., St. Albans. Pp. 172.

The reader of this report will be gratified to find that the research staff of the Rothamsted Station is continuing to make substantial additions to our knowledge in the fields of soil and plant science. The report for 1930 contains information on the organization as well as on the activities of the station. The list of staff members, of temporary workers, and of the publications of the station supplement similar information found in earlier reports. The results of the studies reported for the year relate to field experiments with cereals and other crops; effects of farmyard manure; a new continuous experiment; rotation I; effect of weather conditions on fertilizer efficiency; grasslands; accuracy of field experiments; soil microorganisms; cultivation of the soil; utilization of results of agricultural experiments; composition of the soil—soil analysis; composition of crops; the plant in disease; the farm—geological survey; field experiments at outside centers; fungous diseases in crops at Rothamsted and Woburn; observations on insects attacking farm crops; Rothamsted farm report; and microbiological observations.

The following sections of the report include summaries of papers published in 1930 pertaining to scientific, technical, and other papers; Woburn Experimental Farm; yields of experimental plots, 1930; trustees and committee of management—Lawes Agricultural Trust; and society for extending the Rothamsted experiments.

Results of Vegetation and Laboratory Experiments. The Fifteenth Annual Report (Russian) of the Agricultural Chemical Experiment Station, Petrovsko-Razumovskoye, 1930. By D. N. PRIANISHNIKOV. Published by the Agricultural Chemical Experiment Station of the Timeryazev Academy, Petrovsko-Razumovskoye, U. S. S. R., 1930. Pp. xii + 544, figs. 145.

The present report includes some of the work of the station for the years 1927 and 1928. A short introduction and a list of staff members and collaborators are followed by a series of papers whose titles are given in the following. Each paper is followed by a short résumé in German. The list of authors and titles of these papers follows: S. N. Rosanow and E. K. Lukownikow—"Thermophosphate aus dem Chibiner Apatit-Nephelin Gestein"; W. M. Kletschkowsky—"Ueber die Konstanz und über die Verschiebung der Wirkungsfaktoren in der Ertragsformel von Mitscherlich"; A. G. Schestakow—"Ueber die Rolle des Kalziums bei der Assimilation von Eisen durch die

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J. G. Lipman.

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